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The Isolation and Properties of Deoxyribonucleoprotein Particles Containing Single Nucleic Acid Molecules

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(*Received 22 September 1958*)

Summary

A gel-free solution of deoxyribonucleoprotein was prepared in high yield from calf thymus by maintaining the concentration and ionic strength at very low values and by careful homogenization. The resulting material was examined by a number of different macromolecular methods and found to consist of completely dispersed deoxyribonucleoprotein particles of high asymmetry and containing nearly equal amounts of deoxyribonucleic acid and protein. The molecular weight determined by light scattering was 18·5 million. Upon dissociation the deoxyribonucleic acid obtained had a molecular weight of 8 million suggesting that the deoxyribonucleoprotein particles consisted essentially of one deoxyribonucleic acid molecule and a nearly equal amount of protein. Hydrodynamic investigations and direct electron microscopic examination indicate that the protein is rather evenly distributed along deoxyribonucleic acid chains. Optical rotatory dispersion and infrared spectral studies, while ambiguous, do suggest that a major part of the protein is in the α-helical configuration. It is shown that a large fraction of the histone can be accommodated in sections having the α-helical configuration in the large groove of the double-stranded deoxyribonucleic acid helix and that such a model is consistent with the observations made thus far.

While the structure and molecular properties of deoxyribonucleic acids (DNA) have been elucidated in some detail during the last decade and the nature of histones has been greatly clarified, the way in which these two types of molecules are combined in the nucleoprotein complex or chromosome has remained obscure. Since the nucleoprotein (DNP) can be obtained as an aqueous solution by dispersing isolated chromosomes directly in water it would appear that the molecular characterization of the nucleoprotein in this solution would provide important evidence concerning the structure of the conjugated compound and from this, information concerning the building units in the chromosomes might be deduced. However, numerous investigations of the DNP in aqueous solutions have indicated a very complex behaviour that defied simple analysis and suggested that it was actually a gel-like network devoid of clearly identifiable subunits.

In taking up this problem we have re-examined the individual steps involved in preparing this aqueous solution from the source most often studied, calf thymus, and have been able to modify the procedure so that nearly all of the DNA in the original cells is obtained as discrete, completely dispersed and soluble nucleoprotein particles which contain single DNA molecules (a preliminary summary of this work has been published; Doty & Zubay, 1956).

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Earlier Preparations and Characterizations of Nucleohistone (Calf Thymus)

The most used preparative method, described by Chargaff (Crampton, Lipschitz & Chargaff, 1954; Chargaff, 1955) is the result of successive modifications by Hammarsten (1924) and others (Mirsky & Pollister, 1942; Gajdusek, 1950; Stern, Goldstein & Albaum, 1951; Bernstein & Mazia, 1953; Shooter, Davidson & Butler, 1954) of procedures developed at the turn of the century. Its essential steps are as follows. (1) Fresh calf thymus is homogenized briefly with saline-citrate (0·10 M–NaCl and 0·05 M–sodium citrate at pH 7) and the sediment recovered after centrifuging. (2) The sediment is suspended in saline-citrate and centrifuged again. (3) The sediment is suspended and centrifuged three times in water in order to remove electrolytes, at which point it begins to become gelatinous corresponding to the swelling of the chromosomes. (4) The gelatinous sediment is blended or shaken with water to produce a homogeneous, very viscous phase or gel containing 5 to 10 mg/ml. of nucleoprotein. (5) The nucleohistone is usually precipitated with 0·15 M–NaCl, washed with water and lightly dried or lyophilized.

While variations in different steps do clearly influence the end product, in all cases reported it is so viscous and gel-like as to prevent investigation by the usual macromolecular methods. Of course this state does not interfere with its chemical investigation. When the gel-like viscous solution is diluted with water, swelling occurs but fragments of gel remain and in the ultracentrifuge a gel boundary is seen to form in the middle of the cell (Shooter *et al.*, 1954). In some cases a slower, freely-moving boundary is seen as well but this is generally identified as a breakdown product of the gel resulting from enzymatic attack. Even if discrete and dispersed nucleoprotein particles were obtained, considerable difficulty would be expected in carrying out the measurements of various macromolecular quantitites which depend strongly on concentration. The reason for this lies in the fact that the nucleoprotein particles carry a net negative charge (about one seventh that of DNA; see Davidson & Butler, 1956) and as a consequence require significant amounts of additional salt to eliminate the polyelectrolyte effects on the concentration dependence. However, the nucleoprotein precipitates, even from the gel phase, at concentrations of salt as low as 0·01 M (Shooter *et al.*, 1954). Consequently only a narrow range of ionic strength is left which is below the insolubility threshold and substantially above the counter-ion concentration provided by the sodium ions paired with the negative charges on the nucleoprotein.

Despite these difficulties numerous measurements have been made on this kind of system. A comprehensive and critical summary of these is provided by Davidson, Conway & Butler (1954).

Modification of the Preparative Procedure in Order to Produce Nucleohistone Solutions

Upon examining the gel-like DNP resulting from the preparative procedure described in the previous section it appeared that the cross-linkages between the long DNP units were due to association between portions of the histone from different DNP molecules. The tendency of histones to associate and the ability of urea to dissociate them as well as the DNP gel (Luck, *et. al.*, 1956) supported this view and, as a consequence, we sought to find means of suppressing the formation of these cross-linkages and hence the gelling of the DNP.

By altering each stage of the preparative procedure we were able to conclude that gel formation is favoured by the following conditions: (1) Relatively high DNP

concentration (greater than about 0·08 g/dl.), (2) salt precipitation and permitting the salt-washed DNP to stand before homogenizing in water and (3) by carrying out the water homogenization too slowly. It is clear that all of these conditions encourage either the partial separation of histone from DNP or the interaction of such dangling parts of the histone chains. In addition to eliminating these conditions it was necessary to eliminate enzyme action. This was done by working in the presence of sodium ethylenediamine tetra-acetate to prevent deoxyribonuclease action and to maintain a pH of about 8 to suppress the action of cathepsins on histones (Maver & Greco, 1949). These factors led to the following procedure for isolating DNP in high yield free of gel structure.

The procedure is outlined in Fig. 1. Calf thymus glands, obtained about 10 min after slaughter, were quickly frozen in liquid nitrogen. The frozen tissue was broken

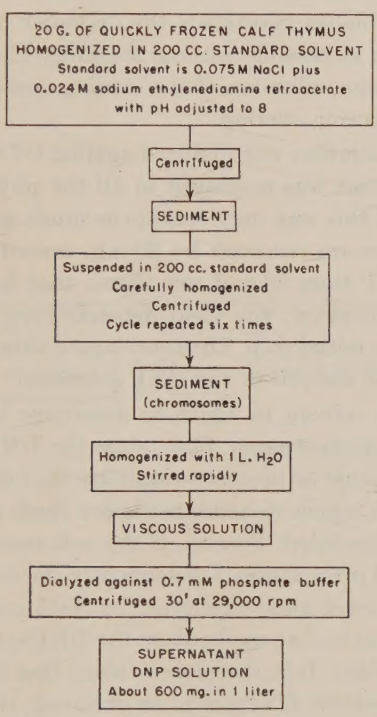

FIG. 1. Outline of preparative procedure for deoxyribonucleoprotein particles.

into small pieces and stored in plastic bags in dry ice until used. Working in a cold room (3 – 5°C.), about 20 g of frozen thymus glands were homogenized in a Waring blendor with 200 ml. of what we shall refer to as saline-versene. It consists of 0·075 M–NaCl and 0·024 M–sodium ethylenediamine tetra-acetate adjusted to pH 8. This solvent inhibits DNaase I by chelating divalent cations and DNaase II by exceeding the pH at which it is active.

Two special steps were taken in the homogenizations. First capryl alcohol was blended with the saline-versene solution just before the tissue or DNP–containing sediments were added. This dispersal of the alcohol greatly improved its suppression of surface denaturation. In the initial homogenization 1 ml. of alcohol was used; in subsequent steps 0·5 ml. The second precaution was the use of a powerstat (variable transformer) in series with the Waring blendor for precise speed control.

The initial homogenization of the tissue with the saline-versene was carried out for 1 min at 80 volts and 4 min at 50 volts. The homogenate was strained through 12 thicknesses of pre-washed cheese cloth to remove connective tissue and then centrifuged in the International Centrifuge at 2000 rev/min. ($\sim$ 400 *g*) for 10 min.

The sediment was again homogenized in the same volume of saline-versene solution for 5 sec at 80 volts and 30 sec at 45 volts. The homogenate was again centrifuged and the sediment homogenized in the same way in the saline-versene solution. This cycle was repeated 6 more times. The final product (sediment) was dispersed by rapid mixing (60 volts) with water (no capryl alcohol was added this time) in the blendor starting with 50 ml. of water and increasing this to 1000 ml. in 5 sec. The mixture was then quickly transferred to a 2 l. beaker and stirred rapidly with a propeller-type stirrer for one hour. It is important that the water be doubly distilled to insure the absence of metal ion contamination. It was found desirable to have the diameter of the glass propeller stirrer blades two-thirds the diameter of the beaker and for the stirring speed to be as high as possible without foaming. The fulfillment of this latter condition requires continuous reduction of the stirring speed because of the decrease in viscosity which occurs during stirring.

After stirring the DNP solution was dialyzed against 0·7 mM-potassium phosphate buffer at pH 6·8; this solvent was employed in all the physical measurements. The DNP solutions prepared in this way may contain as much as 15 % of the DNP in the form of gel particles; these are removed by 30 min centrifugation at 70,000 *g*. The yield is about 0·6 g of DNP from 20 g of wet tissue, that is, about two-thirds of the DNP originally present. However, the yield depends very much on the amount of homogenization used in the initial step. The total lapsed time of a preparation is about 3 hr to the beginning of the dialysis of the DNP solution.

The choice of a suitable solvent in which to determine the solution properties of DNP was limited by two circumstances. First, since the DNP carries a large negative charge at pH 6·8 some salt must be present to stabilize the interparticle repulsions over the concentration range in which measurements are made; otherwise physical data cannot be accurately extrapolated. Second, if the salt concentration exceeds about 0·1 M, DNP aggregates and precipitates. Consequently the onset of aggregation can be expected at considerably lower ionic strengths. At NaCl concentrations above 0·6 M the system is soluble but this is of no use because the DNP is dissociated into DNA and histone (Davidson *et al.*, 1954). It is therefore evident that if results characteristic of completely dispersed and soluble DNP are to be obtained, the measurements must be carried out in the narrow range of salt concentration above the region where polyelectrolyte effects make physical measurements ambiguous and below the threshold of incipient aggregation.

This region was located through the use of viscosity measurements which are very sensitive to both electrolyte effects and aggregation. The pronounced dependence of the reduced specific viscosity of DNP on salt concentration is illustrated in Fig. 2 where the concentration of the potassium phosphate buffer, pH 6·8, was varied. It can be seen that the reduced specific viscosity increases rapidly as the concentration of salt is lowered. This effect is presumably due to the increased strength of the electrostatic repulsions between the highly charged polyanions. Indeed similar behaviour is shown by DNA itself (Conway & Butler, 1954). There is no region over which the reduced specific viscosity shows a constant value and, consequently, it would be difficult to choose a suitable working concentration of salt from these data alone. However, in contrast to the reduced specific viscosity, the intrinsic viscosity of

DNP as a function of buffer concentration does show a plateau region. In Fig. 3 it is seen that this exists from 0·50 to 1·1 mM-phosphate and above this point it gradually decreases. The fall of intrinsic viscosity above 1·1 mM-phosphate is accompanied by a large increase in light scattering at all angles showing that it is due to aggregation. The constancy of the intrinsic viscosity over a significant range of salt concentration

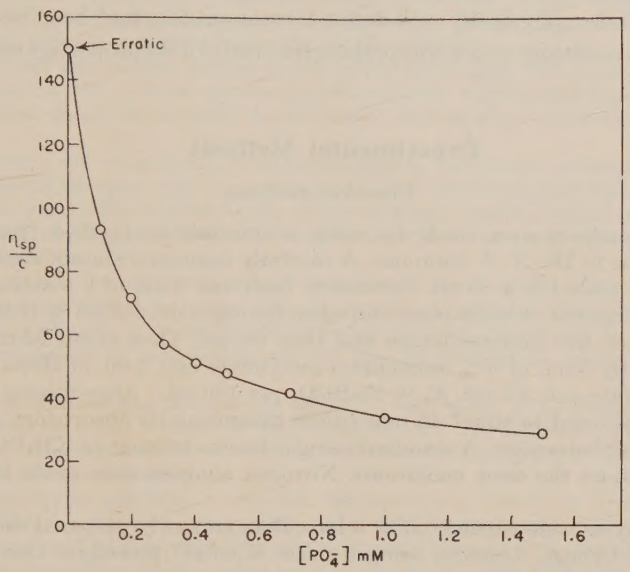

FIG. 2. Reduced specific viscosity of DNP as a function of the concentration of potassium phosphate buffer. (DNP concentration is 6·1 mg/dl.)

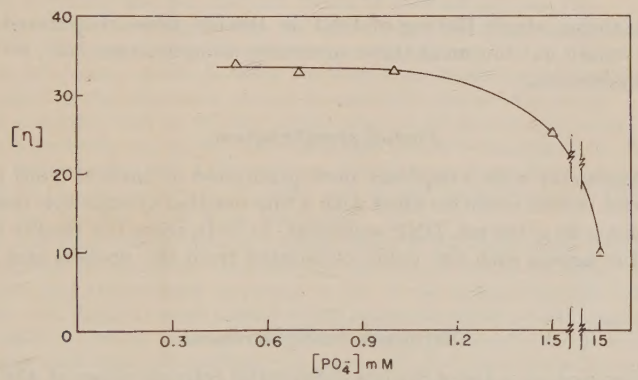

FIG. 3. Intrinsic viscosity of DNP as a function of the concentration of potassium phosphate buffer.

attests to the fact that salt causes negligible aggregation of the DNP below 1·1 mM-phosphate. In view of these results we have chosen 0·7 mM-phosphate buffer, which lies near the middle of the plateau region in Fig. 3, as the working solvent for our physical measurements. Using these conditions it was found that solutions of DNP underwent less than a 5 % change in viscosity over a period of 10 days at 25°C. In addition to the constancy of viscosity with time we observed essentially the same value for the intrinsic viscosity in over 10 different preparations of DNP indicating that we had a reproducible as well as a stable product.

A number of the DNP preparations were also characterized in the analytical ultracentrifuge. From these results, which are discussed later, it was clear that the distribution of sedimentation constants was single peaked and considerably narrower than that observed in DNA itself.

These observations were sufficient to demonstrate that the DNP solutions were reasonably stable and that the hydrodynamic properties of different preparations were reproducible. Thus a physically well-defined nucleoprotein had been isolated by using only very mild conditions and a comprehensive study of its properties seemed justified.

Experimental Methods

Chemical analyses

Phosphorus analyses were made by using a colorimetric method (King, 1932) with modifications due to Dr. N. S. Simmons. A carefully measured aliquot containing roughly 4 mg of DNP is placed in a 50 ml. volumetric flask and 3 ml. of 9 N-sulfuric acid added. The mixture is digested on a hot plate and when fuming a drop of 30 % H_2O_2 is added. The sample is digested five minutes longer and then cooled. Then about 35 ml. of water are added followed by 5 ml. of 5 % ammonium molybdate and 2 ml. of Metol (1 g *p*-methylaminophenolsulfate and 8·6 ml. 35 % $NaHCO_3$ per 100 ml.). After mixing it is diluted to the mark, and allowed to stand 45 min before measuring its absorbance at 750 mμ in a Beckman spectrophotometer. A standard using a known amount of KH_2PO_4 is run simultaneously and under the same conditions. Nitrogen analyses were made by the Kjeldahl method.

Nitrogen analyses were carried out at a later date on newly prepared samples by M. R. Watson (King's College, London) using a micro-Kjeldahl procedure that gave accurate quantitative results on purified nucleotide samples.

Dry weight determinations

Solutions containing about 100 mg of DNP in 100 ml. were evaporated in a weighing bottle of known weight in vacuum at three successive temperatures, 25°, 60° and 110°C, in order to avoid hydrolysis.

Partial specific volume

A simple pychnometer with a capillary neck graduated in units of 0·001 ml. and having a volume of 100 ml. which could be filled with a fine-needled syringe was used for measurements on 0·15 and 0·30 g/100 ml. DNP solutions. In both cases the results yielded a value of 0·68 ml./g. This agrees with the value calculated from the protein and DNA contents of the DNP.

Refractive index increment

This was determined on a Brice-Speiser differential refractometer at 436 mμ and found to be 0·193.

Intrinsic viscosity

This was determined in the manner previously described (Reichman, Rice, Thomas & Doty, 1954) in which time of flow is measured in a three-bulb viscometer yielding results in the gradient range of 50 to 250 sec^{-1}.

Measurement of concentration

Simultaneous measurements of the absorbance at 259 mμ and the dry weight led to a value of 106 $\pm$ 5 for the extinction coefficient for a solution containing 1 g/dl. of DNP. This value was the basis of concentration determinations throughout.

INSTRUCTIONS TO AUTHORS

1. The *Journal of Molecular Biology* will publish papers on the nature, production and replication of biological structure at the molecular level, and its relation to function. Suitable subjects are sub-cellular organization: molecular genetics: structure and replication of viruses: molecular structure of muscle, nerve and other tissues: structure of proteins, nucleic acids, carbohydrates, lipids, etc., and their synthetic analogues, as investigated by X-rays, light absorption and other methods: problems of inter- and intra-molecular energy transfer.

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Papers should contain the maximum density of information compatible with clarity, and every sentence should be scrutinized to ensure that its presence is essential. Authors should include enough experimental details to enable others to repeat the work (these may be printed in small type). Careful preparation of manuscript will aid prompt publication; carelessness will cause delay.

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A short running title of not more than 40 characters (including spaces), suitable for page headings, should be indicated if the full title is longer than this.

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The abstract should be headed as follows: title of paper; name(s) of author(s); *J. Mol. Biol.*, followed by space for the year, volume and page references; abbreviated postal address (including country of origin) to which applications for reprints may be sent. For example: The isolation and properties of deoxyribonucleoprotein particles containing single nucleic acid molecules. G. Zubay & P. Doty. J. Mol. Biol. . . . . (Dept. of Chem., Harvard Univ., Cambridge, Mass., U.S.A.). —text of abstract follows.

10. *Reprints.* Fifty reprints of each paper will be supplied free of charge; additional copies may be purchased.

Light scattering

Light scattering measurements were made in a Brice-Speiser photometer at 436 mμ using an Erlenmeyer flask-shaped cell (Reichman *et al.*, 1954). Solvent was clarified by filtering through an ultrafine sintered glass filter directly into the light scattering cell. Solutions were clarified by ultracentrifugation of a 0·03 g/dl. solution for 30 min at 29,000 rev/min in a Spinco medium sized rotor. Under these conditions the small percentage of aggregates which exist in the solution are efficiently spun out (Boedtker & Doty, 1956). The scattering was measured at various concentrations by adding the clarified solution with a pipette to the solvent in the light scattering cell. The scattering was usually measured at three or four concentrations and the final concentration in the light scattering cell was then determined spectrophotometrically.

Sedimentation

Sedimentation analyses were made in a Spinco Model E Ultracentrifuge at room temperature and corrected to 20°C. Schlieren optics were used down to a concentration of 0·015 g/dl. DNP. In addition, several experiments were performed at 0·006 g/dl. DNP with an ultra-violet absorption optical system. The optical density variation on the film was transferred to height *versus* distance curves on centimeter graph paper with the Spinco Model R Analytrol in such a way that the optical density of the original solution is roughly proportional to the height of the reading on the graph paper. The rate of movement of the point of maximum slope on different frames was measured because this quantity is directly comparable with the schlieren peak regardless of any polydispersity effect. The $S^0_{20,w}$ was obtained from a plot of $1/S_{\bar{z}0,w}$ against concentration. The plot was linear over the range of concentrations studied.

Flow birefringence

The extinction angle, χ, and the birefringence, $\delta\eta$, were measured on a Rao instrument over a gradient range from 150 to 2000 sec^{-1} in a room thermostated at 25°C. The concentration dependence of χ and $\delta\eta/c$ were small over the range of concentrations studied.

Infrared spectroscopy

The infrared measurements on DNP were made with the help of Drs. E. R. Blout and H. Lenormant on a sample of DNP exhaustively dialyzed against water and then centrifuged to a pellet at the bottom of a centrifuge tube. The pellet was distributed over the surface of an AgCl window and allowed to dry at 52 % relative humidity. The infrared spectrum was measured on a Perkin-Elmer double beam instrument in the region 1000 cm^{-1} to 2000 cm^{-1}.

Electron microscopy

The electron microscopy was carried out in Dr. Cecil Hall's laboratory by a new technique which he has recently developed (Hall, 1956; Hall & Litt, 1956; Hall & Doty, 1958) involving the use of freshly cleaved mica as a substrate. Materials are sprayed onto the surface of the mica from a commercial throat sprayer. An aqueous suspension of polystyrene spheres of 880 Å diameter is added to the solution to aid in locating the droplets, focusing and calibrating. Other details of the method are provided in the references just quoted.

The Macromolecular and Chemical Properties of Deoxyribonucleoprotein

Chemical composition

Phosphorus and nitrogen analysis on several samples yielded 4·55 and 16·8 as the respective percentages present. This sets the N/P ratio as 3·70 as previously reported (Doty & Zubay, 1956) with an uncertainty of $\pm$ 0·2. Further measurements on later preparations carried out in collaboration with M. R. Watson yielded 4·48 and 16·8 for

the percentages of phosphorus and nitrogen and this in turn leads to 3·77 for the N/P ratio. The uncertainty remains about the same, ± 0·2. For these analyses the sodium ethylenediamine tetra-acetate was omitted from the standard solvent in the two final washings (to eliminate N contamination) and water replaced the buffer in the dialysis (to avoid P contamination). These later measurements were made as a check on our earlier results in view of the difference between them and the lower results, 15·7 per cent N (Davidson & Butler, 1956; Steiner, 1952) and 3·7 (Davidson & Butler, 1956) and 4·4 (Steiner, 1952) per cent P, reported by others on materials prepared differently.

In order to assess the homogeneity of the preparation with regard to composition a 0·02 g/dl. solution, dialyzed against water, was centrifuged until 60 % in one case and 90 % in another case had been removed from the supernatant. The analysis of the supernatant gave the same results, the values of the N/P ratios being 3·7 and 3·6 respectively. Thus there appears to be no significant heterogeneity with respect to gross chemical composition in the DNP.

To compute the DNA content of the DNP from these results the phosphorus content of the sodium salt of DNA, 9·29 %, must be adjusted for the loss of about 86 % of the sodium in the DNP (Davidson & Butler, 1956). On this basis the phosphorus content of DNA becomes 9·6 %. Using this together with the phosphorus content of the DNP leads to a DNA content of 47 ± 2 % in the DNP on a weight basis.

Accepting 47 % as the DNA content of DNP and taking 18·5 % as the average of the nitrogen content of DNA reported in the literature, one calculates 17·0 % nitrogen for DNP. This is in satisfactory agreement with our experimental value of 16·8 %.

Light scattering

Because there is some uncertainty in deriving a molecular weight from sedimentation constant and intrinsic viscosity measurements on DNA (Doty, 1957) the same difficulties may be expected here. Consequently we were forced to rely heavily on light scattering for a value of the molecular weight of DNP. Accordingly centrifugal clarification was always carried out at concentrations below 0·03 g/dl. to insure effective removal of larger particles (Boedtker & Doty, 1956). Six light scattering determinations on three different preparations were carried out. A typical result is plotted in Fig. 4 and the results are summarized in Table 1. The amount of DNP

Table 1

Light scattering and viscosity measurements of DNP

Sample Number	% DNP removed	Molecular Weight	$(\overline{r_z^2})^{1/2}$	$[\eta]$
11	47	25,000,000	3,800Å	35
11	54	18,000,000	3,900	35
13	21	18,500,000	4,600	33
13	21	18,500,000	4,000	—
15	20	18,500,000	4,000	35
15	38	19,200,000	4,300	33

removed during the centrifugal clarification is included. The average molecular weight is seen to be about 18·5 million excluding the first measurement from the average because of its rather large deviation. While the reproducibility of this figure is good its precision is subject to further uncertainty because of the possibility of systematic error in the extrapolation (Doty, 1957). This source of error disappears when the

molecular weight distribution is essentially that of the most probable distribution and since this turns out to be nearly the case for calf thymus DNA (Doty, 1957; Litt & Doty, unpublished) the additional uncertainty in this case is probably not more than 10 %.

The average of the measurements of the radii of gyration was 1700 Å. If the model of a randomly coiled chain is assumed for DNP this value can be interpreted as the root-mean-square of the z-average of the end-to-end length, $(\overline{r_z^2})^{1/2}$, with a value of 4170 Å.

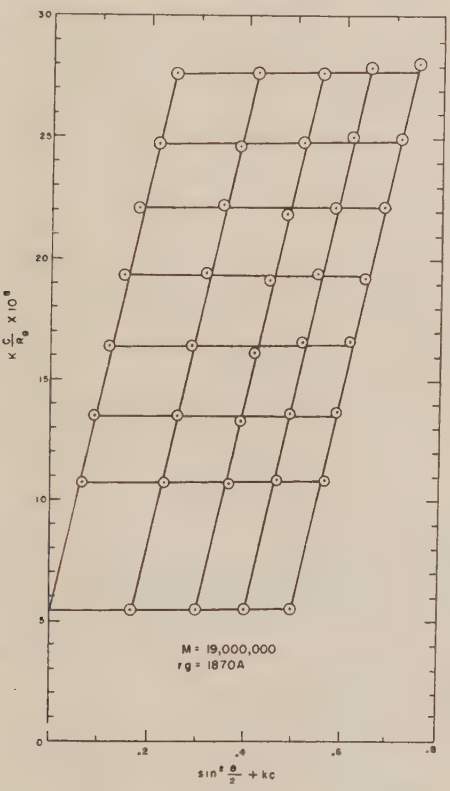

FIG. 4. Light scattering plot for DNP.

Making use of 8 million as the average molecular weight of calf thymus DNA from light scattering (Doty, 1957) and the result of the previous section, 47 % by weight of DNP is DNA, the molecular (or particle) weight of DNP composed of one molecule of DNA would be 17 million. The proximity of this to the mean of the measured values, 18·5 million, clearly suggests that the DNP particles consist of single DNA molecules and a protein complement of approximately the same weight.

To confirm this, DNP was precipitated from solution by addition of NaCl, and DNA was prepared from it according to the Simmons B method (Rice & Doty, 1957). Light scattering measurements on the product did indeed yield a molecular weight of 8 million.

Intrinsic viscosity

In Fig. 5 are shown the measurements leading to the determination of the intrinsic viscosity of one sample of DNP. The results of other measurements are shown in Table 1. From this a mean value of 34 in units of dl./g is obtained. The value of k' in

the Huggins equation (Huggins, 1942) for the data in Fig. 5 is 0·75. This value is at the upper limit observed for polymers and suggests that there are strong attractive interactions between the DNP molecules in the 0·7 mM-phosphate buffer.

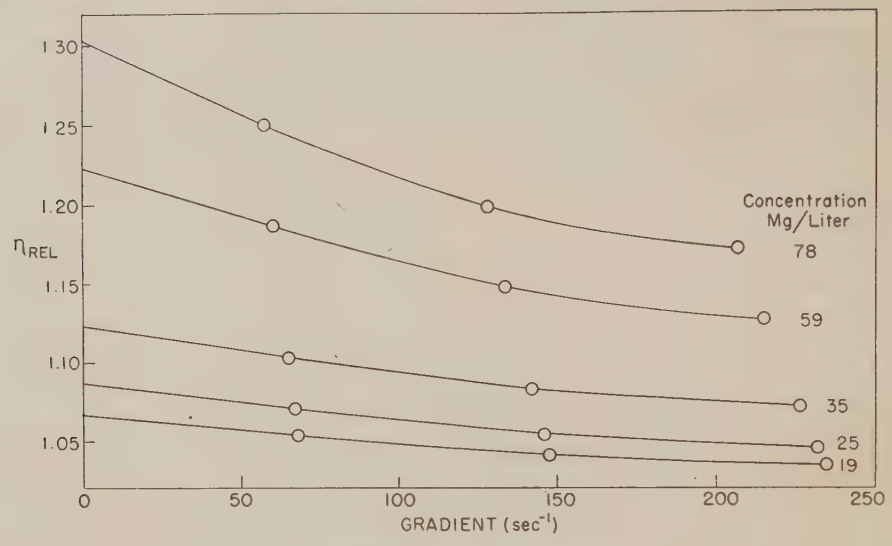

FIG. 5. The determination of the intrinsic viscosity of DNP.

Sedimentation

The sedimentation rate corrected to 20°C. in water was determined at several concentrations for several preparations. A typical set of results together with one determination at 0·0060 g/dl. using ultraviolet optics is presented in Fig. 6. The intercept yields a value of 48 S for $S^0_{20,w}$: the mean of all determinations was 50 S. A line showing the concentration dependence for DNA is shown in Fig. 6 for comparison.

As a test of homogeneity, a 0·03 g/dl. solution of DNP was centrifuged until 20 % in one case and 40 % in another had been removed. The supernatant solutions were then diluted to 0·006 g/dl. and their sedimentation patterns determined with ultraviolet optics. The mean sedimentation constant was the same as that measured for the uncentrifuged solutions, namely 36 S. This indicates that there are no substantial contaminants with sedimentation constants considerably higher than the mean value.

The ultraviolet absorption sedimentation diagrams for DNP were considerably sharper than those observed in this Laboratory for DNA in 0·2M-NaCl. A crude index of the breadth of the distribution of sedimentation constants can be readily obtained by taking the ratio of the sedimentation constant at 80 % absorption and dividing by that at 20 % absorption. For calf thymus this ratio is about 1·8 (Litt & Doty, unpublished). For DNP it is about 1·3. Since the molecular weight dependence of sedimentation constant is not known for DNP this result cannot be interpreted in terms of molecular weight distribution.

Flow birefringence

The extinction angle and birefringence of flow of DNP solutions in the range of 0·03 g/dl. were measured as a function of gradient. To provide comparison similar measurements were made on DNA solutions in 0·15 M-NaCl. The extinction angle results together with the "lengths" of hydrodynamically equivalent ellipsoids are shown in Fig. 7. The birefringence results are presented in Fig. 8.

From Fig. 7 it is seen that either the size of DNP is much smaller than that of DNA or it is not as well oriented at the gradients obtained. However, in Fig. 8 it is clear that the birefringence has reached its maximum in this gradient range and, since DNP can not be considered to be a very flexible particle, it must be concluded that the orientation is high, indeed more nearly complete than with DNA. Consequently, we

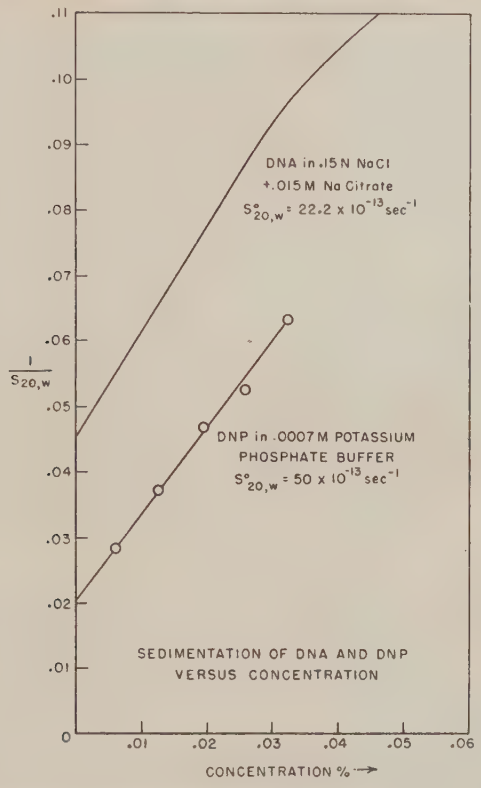

Fig. 6. The determination of the sedimentation constant of DNP. (The upper line shows the behaviour of DNA for comparison.)

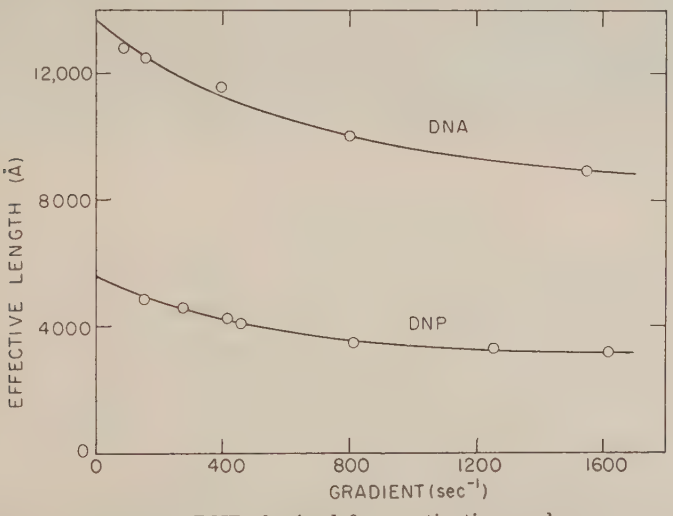

Fig. 7. The effective length of DNP obtained from extinction angle measurements as a function of gradient.

can conclude that the maximum linear dimension of DNA is about double that of DNP. This conclusion is similar to that obtained from light scattering measurements.

If the measurements on DNA were carried out to higher gradients where orientation would be maximal, the range of lengths would be larger and comparable to those observed for DNP. Consequently, we can draw the tentative conclusion that the dimensional polydispersity of the DNP is of the same order as the DNA.

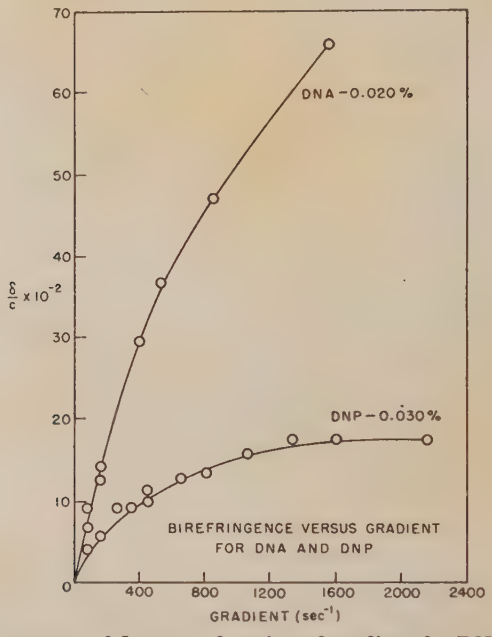

FIG. 8. Birefringence of flow as a function of gradient for DNP and DNA.

The birefringence of flow of DNP solutions is of the same sign as DNA, that is negative, but it is only about one-fifth as great. There are probably two reasons for this. Since the DNP particle is considerably less extended in space, but presumably of the same overall contour length, it will not have as many base pairs perpendicular to its direction of alignment as does DNA. In addition to this an effect will be expected from the protein if the peptide bonds have a preferential orientation relative to the DNA chain. The present data do not allow a resolution of the relative effect of this second contribution: it must, however, be relatively minor since the birefringence of the peptide bond is so much weaker than that of the nucleosides.

Electron microscopy

Electron micrographs taken in collaboration with Professor C. E. Hall (Hall, 1956; Hall & Litt, 1956; Hall & Doty, 1958) are shown in Plate 1. The DNP was sprayed as an aqueous solution and the DNA from an ammonium carbonate-ammonium acetate buffer. The electron micrographs of DNP show the same general features as those of DNA, that is, they show principally long thin structures which are only slightly coiled provided the concentration of spraying is sufficiently reduced so as to prevent intermolecular interferences during drying. At the ends of DNP particles a circular ring super-imposed upon the main cylindrical region is often seen but we can attach no significance to this at present. When diameters are measured for those cases that are oriented perpendicular to the shadows, values of about 20 Å are ob-

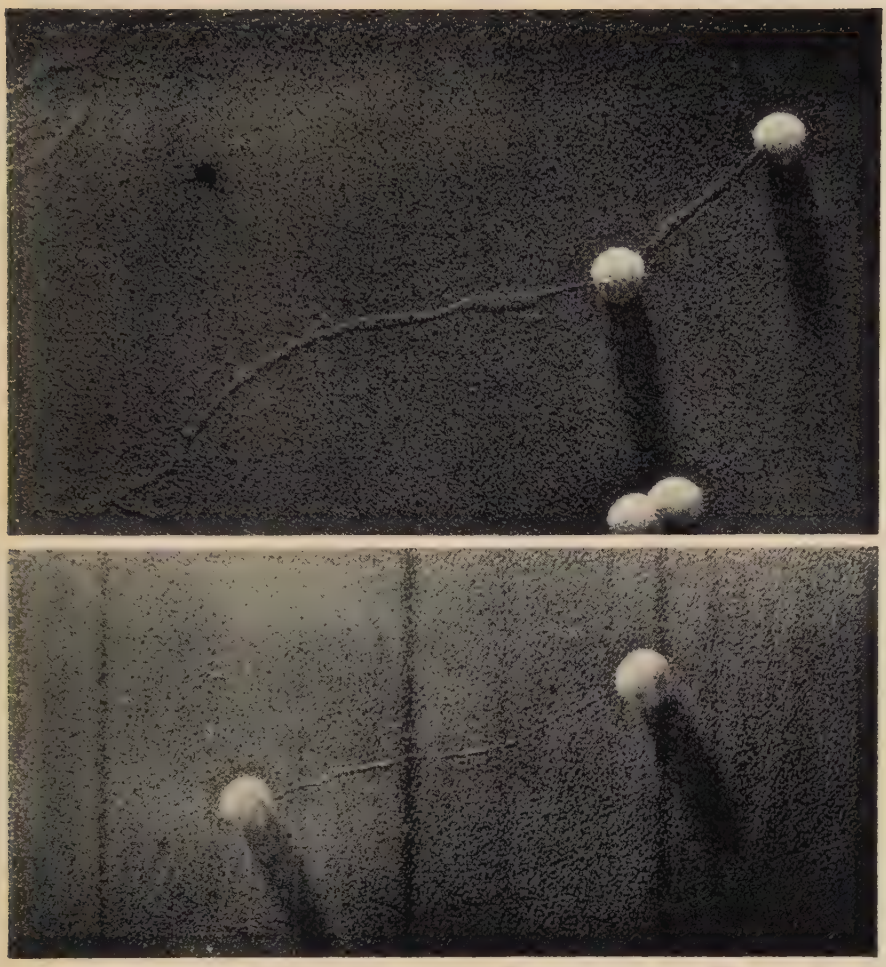

PLATE I. Electron micrograph of DNP (above) and DNA (below).

[*To face page* 12.

tained for DNA and about 30 Å for DNP. Occasionally one finds a 20 Å thick region in DNP but this is almost always adjacent to a region that is 40 Å or more in thickness and usually lies adjacent to a polystyrene sphere. This suggests that the histone may have been laterally displaced by interaction of the DNP and the polystyrene sphere during drying.

Although not definite in themselves these electron microscopic observations provide strong support for the view that the histone is spread rather uniformly over the double stranded DNA structure in a manner consistent with a doubling of the mass-to-length ratio relative to DNA.

Conclusions regarding the size and shape of DNP

The light scattering, hydrodynamic and electron microscopic evidence permit the drawing of a fairly clear picture of the DNP molecules in solution. However, the result is not fitted by any of the geometrical models that suffice to describe many simpler macromolecular substances and, as a consequence, the shape and spatial extension of DNP cannot be described in terms of the numerical value of a single parameter characteristic of a given model.

The DNP particles are composed of single DNA molecules surrounded for the most part by an equal weight of histone. If the DNA was in the form of an uninterrupted Watson-Crick helix its length would be about 40,000 Å. However, the DNP particle in solution is confined to a domain whose greatest dimension is only about one-tenth of this value. Since corresponding DNA occupies a considerably larger volume (the maximum dimension being about 7000 Å) it must be concluded that the DNP particle is more contracted than if the protein were not present. This could be due either to a greater degree of natural coiling made possible by the neutralization of so much of the negative charge by the protein or to the crosslinking of the DNA backbone at a number of points through histone-histone interaction. Since the DNA structure appears to be preserved at radii of curvature as small as 400 Å (Hall & Doty, unpublished) the convolutions required for the degree of contraction observed here would not require local breakdown of DNA structure.

If the DNP particles were somewhat internally crosslinked the particles would not behave hydrodynamically as homologous, randomly-coiled chains. Inasmuch as the hydrodynamic properties do not fit such a model this negative evidence is consistent with a modification such as that produced by intramolecular cross-linkage. The failure of the simple model to fit is most marked in terms of intrinsic viscosity. The end-to-end length from light scattering, which is consistent with the flow birefringence measurements as well, would predict in terms of the Flory-Fox relation (Flory & Fox, 1951) an intrinsic viscosity of only 4 compared to the measured value of 34. Thus DNP joins denatured DNA (Doty, 1957; Rice & Doty, 1957; Ehrlich & Doty, 1958) in failing to behave hydrodynamically as an extended random coil.

Further Experiments Relating to the Structure of the DNP Particles

In this section we report a few preliminary experiments which relate to the manner in which the DNA and protein are united in DNP.

Infrared spectroscopy

It has been well established in a number of cases that under certain conditions the peptide carbonyl stretching frequency (amide I band) of a polypeptide chain is located

at about 1655 cm^{-1} when the chain is in the α-helical configuration and at about 1630 cm^{-1} when it is not (see, e.g. Ambrose & Elliott, 1951; Bamford, Elliott & Hanby, 1956). We have therefore examined films of DNP prepared by evaporating a solution at 52 % relative humidity and room temperature. It is known that DNA alone has a broad band in the 1660 cm^{-1} region and consequently only a qualitative result could be expected. In the spectra of DNP shown in Fig. 9 it is seen that the 1660 cm^{-1} band is strong and broad but free of any significant shoulder at 1630 cm^{-1}. By contrast the infrared spectrum of nucleoprotamine does show a shoulder at 1630 cm^{-1} and dichroism studies have led Ambrose and Butler (1953) to conclude that the protein is in the extended β-configuration in this case.

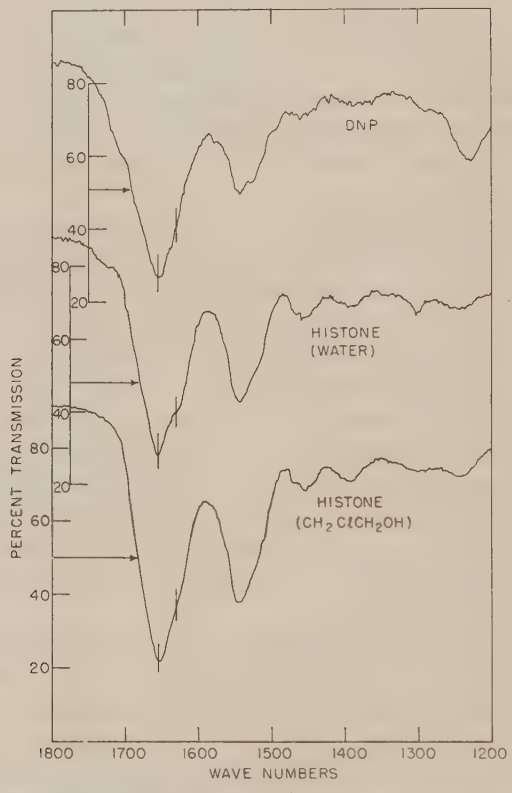

Fig. 9. Infrared spectra of films of DNP (above), histone cast from water (middle), and histone cast from 2-chloroethanol (below). The vertical lines are located at 1630 and 1655 cm^{-1}.

In DNA the 1660 cm^{-1} band is of the same intensity as that of 1220 cm^{-1} (due to the phosphate group); consequently, it is clear that about half the absorption seen in the 1660 cm^{-1} band in Fig. 9 is due to the protein component. When films of histone are examined a similar band is observed at 1660 cm^{-1}, as shown in Fig. 9. If the film is cast from chloroethanol, a solvent strongly favouring the helical configuration (Ambrose & Butler, 1953), no shoulder is seen at 1630 cm^{-1}. However, upon evaporation from water, in which the helical content is only about 20 % (Ambrose & Butler, 1953), a distinct shoulder can be seen at 1630 cm^{-1}. These observations tentatively suggest that the histone is largely in the helical form in thymus DNP. But until a much more thorough study is made of model systems and of the effects of mixing of DNA and histone this must remain only a tentative suggestion.

Optical rotation and rotatory dispersion

Since optical rotation and rotatory dispersion have been shown to be quantitatively related to the helical configuration of polypeptides and proteins (Doty, Imahori & Klemperer, 1958; Yang & Doty, 1957) it was of interest to see if evidence could be found in this way for the existence of the helical configuration in the histone as it exists in DNP. We may consider first results obtained at 5460 Å, a wavelength close to the sodium line where it is known that specific rotations near $-100°$ refer to non-helical configurations and that the completely helical configuration corresponds to values in the range of 0° to 20°. Since DNA is optically active itself its specific rotation must also be determined and the specific rotation due to the histone obtained from the following relation:

$$[\alpha]_{\mathrm{DNP}} = 0{\cdot}47[\alpha]_{\mathrm{DNA}} + 0{\cdot}53[\alpha]_{\mathrm{PROTEIN}}.$$

In assuming this additivity of rotatory power, the possible interaction effects between the DNA and histone have, of course, been ignored. The first difficulty arose in finding that the rotation of DNA was ionic strength dependent, being + 104·8° at 2·5 M-NaCl; + 109·7° at 0·15 M-NaCl and + 162·0° at 0·0015 M-NaCl. It seemed likely that this dependence was due to increasing electrostatic strain within the molecule as the ionic strength was lowered. Since most of the charge in DNP is cancelled by the histone it seemed best to use the values for DNA in high salt. (This choice also minimized the estimated amount of helical configuration.) DNP in 0·7 mM-phosphate buffer was found to have a specific rotation of + 20·7°. Using the above relation the $[\alpha]_{\mathrm{PROTEIN}}$ is found to have a value of − 53·9°. This result suggests that about half of the histone is in the helical configuration.

An attempt was made to extend this work further by measuring the rotatory dispersion. It was only possible, at the time, to measure at four other wavelengths, 3660, 4050, 4360 and 5770 Å. Plotting in such a way as to derive from the slope the value of b_o, it was found that the data fell in a curved plot such that a slope could not be assigned. If, however, the data were fitted with a straight line, the least squares fit gave a value of b_o of − 400: this is equivalent to about 65 % helix.

Examination of histone in chloroethanol (Doty *et al.*, 1958; Yang & Doty, 1957) has shown that in this solvent the helical content is about 70 %; it is likely that the proline content of about 5 % prevents any further increase in helical content.

These observations, like those of the infrared spectra, tentatively suggest that at least half of the histone is in the helical configuration in DNP. Again considerable work remains to be done to provide the basis upon which these results can be more securely interpreted.

Other observations

(1) At salt concentrations above 0·6 M-NaCl, the DNP remained soluble and underwent dissociation. (2) The addition of very small amounts of magnesium ion (10^{-5} M) to the standard DNP solution caused aggregation accompanied by a drop in viscosity. (3) Trypsin and deoxyribonuclease attack their specific substrates in DNP. (4) DNP in the standard solvent underwent thermal denaturation in the range of 80° to 90°C. DNA (calf thymus) at the same ionic strength underwent denaturation at a lower temperature: 60° to 70°C. Thus the histone has a stabilizing effect on the DNA structure. The increase in optical density at 260 mμ attending the thermal denaturation is 40 %.

Molecular arrangement in the DNP particle

The study of the macromolecular properties of DNP, in particular the electron microscopic evidence, indicated that the protein was rather evenly distributed along the intact DNA molecule that forms the core of the DNP particle. Since the infrared spectral and rotatory dispersion studies described in this section suggest that a substantial part of the protein is in a helical configuration it seems appropriate to ask if the histone can be accommodated in this fashion without violating any of the chemical or macromolecular properties.

The surface of the DNA molecule in the Watson-Crick structure contains two grooves of different size and surface composition. The larger of these corresponds to a cylindrical trough of about 10 Å radius (Wilkins, 1957). The possibility of accommodating the histone in this groove as an α-helix was determined with space-filling models. The α-helix used in this construction had the average peptide residue weight of histone of about 103. With such an α-helix it was possible to achieve close-packing of the protein in the large groove when it was on an average radius of 10·5 Å. If the α-helix was to follow the contour of the groove, an amount of protein helix to give a length of 75 Å would be necessary to fit into a complete turn of the DNA helix, that is, in 34 Å along the helix direction. This is calculated from the formula

$$\text{length} = 2\pi\left[r^2 + \left(\frac{\text{pitch}}{2\pi}\right)^2\right]^{\frac{1}{2}}$$

where r represents the distance of the centre of the protein helix from the centre axis of the DNA molecule.

Since the average residue weight of histone is 103 and the translation per residue in the α-helix is 1·5 Å, the mass of histone helix corresponding to a length of 75 Å would be

$$\frac{75}{1{\cdot}5} \times 103 = 5200.$$

For the composition found in this study an amount of protein corresponding to a molecular weight 7400 should be bound per turn of DNA. Since only about 70 % of this amount could occupy the large groove of the DNA as a close fitting α-helix, the remaining 30 % would have to be bound elsewhere, perhaps in the small groove of the DNA as an extended polypeptide chain. It is interesting to note in this connection that thymus histone is divided into two fractions in these relative amounts: Fraction A, 30 % and Fraction B, 70 %. Fraction A contains 28 % (residues per cent) basic residues and 9 % proline whereas Fraction B contains 23 % basic residues and 4 % proline.

Of course, the α-helix does not have the flexibility required to permit it being wound around the DNA in the manner just assumed. Instead one would imagine that contact with the surface of the DNA groove could be maintained, with the helix structure intact for a distance of 15 to 20 Å along the α-helix. Then a break in the helix would be necessary to permit the next helical region to make maximal contact. This frequency of breaks in the α-helical structure corresponds approximately to the combined frequency of the occurrence in histone of proline residues which are known to interrupt the α-helical configuration and of the ends of molecules which occur about every 70 residues. The diameter of a model constructed in this manner would average 28 Å, a value that is consistent with the mean diameter of 30 Å estimated from electron micrographs.

Thus it is seen that a structural proposal can be made that is consistent with all of the experimental observations as well as the more questionable interpretations of the infrared and rotatory dispersion. This model is in contrast with that proposed for nucleoprotamine (Feughelmann *et al.*, 1955) where the protein chain was completely extended except for occasional folds or loops. However, it has in common with the nucleoprotamine model a sufficiently definite spatial arrangement to permit an ultimate test by x-ray diffraction techniques.

Some Preliminary Observations on Other Deoxyribonucleoproteins

In order to place the work described above in a wider context it may be useful to summarize briefly some observations we have made on two other deoxyribonucleoproteins, one from *Arbacia* sea urchin sperm and one from salmon sperm.

When sea urchin sperm, isolated from ripe testes, was carried through the same sequence of operations as that used to prepare DNP from calf thymus a similar product was obtained in a yield of about 65 %. In the standard saline-citrate solvent it was found to have a sedimentation constant, $S^0_{20,w}$ of 33 *S* and an intrinsic viscosity of 25. Although these values are somewhat smaller than the corresponding ones for calf thymus DNP it is clear that similar discrete DNP particles have been obtained from this quite different source.

The application of the same preparative procedure to salmon sperm heads, prepared according to Felix, Fisher, Krekels & Mohr (1951), was completely unproductive because the sperm heads were quite resistant to dispersion in water or very dilute salt solutions. Although a number of other conditions lead to the dispersion of the sperm heads, the DNA and protein were always dissociated in the process. It therefore appeared that the DNA and protein were not complexed in the same way as in calf thymus and sea urchin sperm; instead it seemed that the DNA was extensively crosslinked by the protein in such a manner that no subunits of the sperm head existed. Indeed the structural integrity of the salmon sperm heads were found to be preserved after long exposure to deoxyribonuclease. Such exposure did result in extensive degradation of the DNA as judged from comparing DNA prepared from exposed and control sperm heads. Thus, in contrast to the case of DNP in calf thymus, the degradation of DNA does not profoundly affect the gross structural features of the deoxyribonucleoprotein complex observed in the light microscope, However, degradation of the protein component by exposure to trypsin does lead to a disintegration of the sperm head indicating the crosslinking role suggested above for the protein.

Discussion

Although there is a sizable literature on physical chemical studies of DNP there are few points of comparison between this earlier work and that reported here. The principal cause of this situation is the mistaken view, held until about 10 years ago, that molar NaCl extracted native DNP. The precipitates obtained upon diluting such solutions with water consists of the DNA and protein components of the DNP but the native structure has been destroyed since nearly complete dissociation had occurred in the 1M-NaCl solution. When such material was examined in concentrated salt solution the macromolecular properties observed were often those that can now be recognized as those of DNA. When it became clear that DNP had to be extracted, purified and studied without exceeding very low concentrations of salt (Petermann

& Lamb, 1948; Stern, 1949) interest appeared to slacken. Indeed in the last decade the extensive investigation of this problem is limited to the work of Steiner (1952) and Davidson, Conway & Butler (1954). Steiner's material bears little relation to our DNP with respect to macromolecular properties although the composition is essentially identical. All of the properties he measured, viscosity, sedimentation, light scattering and flow birefringence, were much smaller than we observed. Moreover his reported absence of gradient dependence in viscosity and of concentration dependence in sedimentation is inconsistent with the magnitude of the observed values and cast considerable uncertainty upon the self-consistency of this work. In any event his yield of DNP was so small that it is unlikely that the material was at all representative.

Indeed Steiner's material appears to correspond to a minor component obtained in the study of Shooter *et al.*, (1954). In this latter study the principal component was a thick, gel-like, opalescent material which did not display the properties of discrete units and therefore its study did not yield the kind of data reported here. Since other relevant studies have been evaluated in a recent review (Davidson *et al.*, 1954) there is no need for further discussion on this point.

In conclusion we turn to the hazardous question of how the uniquely defined DNA-protein complex described here might be related to the chromosome structure from which it was removed. Having passed from isolated chromosomes at an early stage of the preparation to DNA derived from the DNP, it would appear that the DNP itself represents a first step in the progression from isolated DNA back to the complex chromosome structure. Alternatively, since the DNP described here is stable with respect to all the steps of the isolation procedure it is reasonable to assume that the complex existed in the chromosome structure. Fortunately this conclusion is open to verification by x-ray diffraction studies.

Further consideration of this point leads to examining the extent to which the DNP isolated here accounts for the known composition of chromosomes.

Mirsky & Ris (1951) report that isolated chromosomes, which correspond to the second stage of our procedure, contain 39 % DNA in the case of calf thymus. Allowing 1 % RNA and 2% lipid, the remainder, 58 %, is protein. If the same amount of RNA and lipid is retained in our preparation the nitrogen and phosphorus analysis would indicate 46 % DNA and 51 % protein. On this basis it would appear that about 7 % of the protein has been lost in the preparative procedure, relative to the DNA.

Mirsky & Ris identified a non-histone protein in the chromosomes and termed it residual protein. This was defined as the protein remaining after extraction of the chromosomes at pH 3 with 1 M-NaCl and enzymatic removal of the DNA. Such protein was insoluble in neutral saline and possesses tryptophan, in contrast to histone. However, it appears that the determination of residual protein in this manner may lead to an overestimate inasmuch as 1 M-NaCl does not bring about the complete dissociation of the calf thymus DNA-protein complex (Shooter *et al.*, 1954; Crampton *et al.*, 1955). In the case of calf thymus, Mirsky & Ris found the residual protein content to be 9 %, one of the lowest values found; taking this as an upper estimate it is clear that this is not a major constituent in the material investigated here. We have not attempted to determine the amount of such protein in our DNP but the ease of complete dissociation to DNA indicated that there was only a very small amount. If this is true, the residual protein would be found in the sediment from centrifuging the DNP solution at the end of the preparation. Thus as a working hypothesis we would suggest that most of the DNA in the chromosome is united with histone in a linear complex such as described here. These units are then held in place in a chromosome

structure formed of other DNA and residual protein. Such a view provides an obvious program for future investigation.

This attempt to suggest the role of this DNP has been described in more detail than it merits at this stage in order to remove misunderstanding introduced by a recent discussion of our preliminary communication (Doty & Zubay, 1956) by Dounce & O'Connell (1958). Some of this misunderstanding is removed by the fuller discussion presented here. Other differences were more sematic than real. These authors have been largely concerned with a "residual protein" that is operationally quite different in definition from that originally proposed by Mirsky and with the union of this non-histone protein with DNA in what they refer to as deoxyribonucleoprotein. By contrast we have focused our attention on the nucleohistone, which together with minor constituents make up what we have labeled DNP. Calf thymus was chosen as a source material since its chromosomes have shown the smallest amount of "residual" protein thus far measured. Thus we have prepared DNP from chromosomes that contain about 90 % nucleohistone and have not considered it expedient at this stage to be concerned with what portion of these minor constituents survive in our DNP preparation.

It is true that we do not take seriously the phenomenon of gelation even though it plays a key role in the work of Dounce. At this stage of investigation of the calf thymus system it appears to us that gelability arises from the mishandling of the material, much like denaturation occurs in mishandling of some proteins, and is therefore something to be avoided. To single this out as a test of native structure seems arbitrary in the extreme. It is not surprising that Dounce & O'Connell found conditions in which a gel could be obtained or that upon long stirring the gel could be broken up into a viscous solution. We avoided this substantial gel formation by the use of blending and stirring under the precisely controlled conditions described. Moreover, the characteristics of the DNP reported here are not those of the redispersed gel described by them. Likewise their concern with the possibility of protease attack on our DNP is essentially misplaced since the scission of occasional peptide bonds will not significantly alter the properties of the DNP we have investigated, whereas such action would greatly modify a DNA-residual protein network visualized by Dounce & O'Connell. It is most important to recognize the evidence for two different deoxyribonucleoproteins, one involving histones principally and the other non-histone proteins. As the elucidation of both of these structures progresses increasingly meaningful questions can be asked about their role in chromosome structure.

This investigation was supported by a research grant (C–2170) from the National Cancer Institute, Public Health Service. One of us (G.Z.) wishes to acknowledge with appreciation a predoctoral fellowship from the National Institutes of Health. In addition we would like to express our indebtedness to Dr. Norman Simmons for advice during the early stages of this work and to Dr. E. R. Blout for help with the infrared spectral studies.

REFERENCES

Ambrose, E. J. & Elliott, A. (1951). *Proc. Roy. Soc. A*, **76**, 208.
Ambrose, E. J. & Butler, J. A. V. (1953). *Disc. Faraday Soc.* **13**, 261.
Bamford, C. H., Elliott, A. & Hanby, W. E. (1956). *Synthetic Polypeptides*. New York: Academic Press.
Bernstein, M. H. & Mazia, D. (1953). *Biochim. biophys. Acta*, **10**, 600.
Boedtker, H. & Doty, P. (1956). *J. Amer. Chem. Soc.* **78**, 4267.
Chargaff, E. (1956). *The Nucleic Acids*, Vol. I, p. 313. New York: Academic Press.

Conway, B. E. & Butler, J. A. V. (1954). *J. Polym. Sci.* **12**, 199.
Crampton, C. F., Lipshitz, R. & Chargaff, E. (1954). *J. Biol. Chem.* **206**, 499.
Crampton, C. F., Moore, S. & Stein, W. H. (1955). *J. Biol. Chem.* **215**, 787.
Davidson, P. F. & Butler, J. A. V. (1956). *Biochim. biophys. Acta*, **21**, 568.
Davidson, P. F., Conway, B. E. & Butler, J. A. V. (1954). *Progr. Biophys.* **4**, 148.
Doty, P. (1957). *J. Cell. Comp. Physiol.* **49**, Supplement 1, 27.
Doty, P., Imachori, K. & Klemperer, E. (1958). *Proc. Nat. Acad. Sci., Wash.* **44**, 424; *J. Amer. Chem. Soc.*, in the press.
Doty, P. & Zubay, G. (1956). *J. Amer. Chem. Soc.* **78**, 6207.
Dounce, A. L. & O'Connell, M. (1958). *J. Amer. Chem. Soc.* **80**, 2013.
Ehrlich, P. & Doty, P. (1958). *J. Amer. Chem. Soc.* **80**, 4251.
Felix, K., Fischer, H., Krekels, A. & Mohr, R. (1951). *Z. Physiol. Chem.* **287**, 244.
Feughelmann, M., Langridge, R., Seeds, W. E., Stokes, A. R., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F., Barclay, R. K. & Hamilton, L. O. (1955). *Nature*, **175**, 834.
Flory, P. J. & Fox, T. G. (1950). *J. Polym. Sci.* **5**, 745.
Flory, P. J. & Fox, T. G. (1951). *J. Amer. Chem. Soc.* **73**, 1904.
Gajdusek, D. C. (1950). *Biochim. biophys. Acta*, **5**, 397.
Hall, C. E. (1956). *J. Biochem. Biophys. Cytol.* **2**, 625.
Hall, C. E. & Doty, P. (1958). *J. Amer. Chem. Soc.* **80**, 1269.
Hall, C. E. & Doty, P. Unpublished results.
Hall, C. E. & Litt, M. (1956). *J. Biochem. Biophys. Cytol.* **4**, 1.
Hamer, D. (1955). *Biol. Bull.* **108**, 3539.
Hammersten, E. (1924). *Biochem. Z.* **144**, 383.
Huggins, M. (1942). *J. Amer. Chem. Soc.* **64**, 2716.
King, E. J. (1932). *Biochem. J.* **26**, 292.
Luck, J. M., Cook, H. A., Eldredge, N. T., Haley, M. I., Kupke, D. W. & Rasmussen, P. S. (1956). *Arch. Bioc hem. Biophys.* **65**, 449.
Maver, M. E. & Greco, A. E. (1949). *J. Biol. Chem.* **181**, 853.
Mirsky, A. E. & Pollister, A. W. (1942). *Proc. Nat. Acad. Sci., Wash.* **28**, 344.
Mirsky, A. E. & Ris, H. (1951). *J. Gen. Physiol.* **34**, 475.
Petermann, M. L. & Lamb, C. M. (1948). *J. Biol. Chem.* **176**, 685.
Reichman, M. E., Rice, S. A., Thomas, C. A. & Doty, P. (1954). *J. Amer. Chem. Soc.* **76**, 3407.
Rice, S. A. & Doty, P. (1957). *J. Amer. Chem. Soc.* **79**, 3937.
Shooter, K. V., Davidson, P. F. & Butler, J. A. V. (1954). *Biochim. biophys. Acta*, **13**, 192.
Steiner, R. F. (1952). *Trans. Faraday Soc.* **48**, 1185.
Stern, K. G. (1949). *Exp. Cell. Res.* **1**, Supplement 1, 97.
Stern, K. G., Goldstein, G. & Albaum, G. G. (1951). *J. Biol. Chem.* **188**, 273.
Tristram, G. R. (1947). *Nature*, **160**, 637.
Wilkins, M. H. F. (1957). *Biochemical Society Symposium*, No. 14, p. 13.
Yang, J. T. & Doty, P. (1957). *J. Amer. Chem. Soc.* **79**, 761.

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The Relationship between the Metabolic Pools of Photosynthetic and Respiratory Intermediates †

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Using radioactive carbon dioxide, an attempt has been made to distinguish the various pools of intermediary metabolism which may be physically or chemically separate within the cell. Some correlation between the structural elements of the cells and these pools appears possible.

1. Introduction

The question of the interrelations at a biochemical level of the processes going on in respiration and photosynthesis has long been of interest. Almost every compound lying in the pathway of the carbon reduction cycle also plays a part in one of the two main routes of respiratory breakdown of sugars, glycolysis and the pentose phosphate cycle. Indeed, part of the carbon reduction cycle involves reactions which are the reverse of those taking place in glycolysis, while the transketolase reactions in the pentose phosphate cycle are the same as those operating in the photosynthetic cycle in the opposite direction.

Once the detailed biochemical reactions included in these cyclic operations became known, the question immediately arose as to what influence these processes had upon each other, and whether identical metabolic reservoirs of the intermediates in the same cell were involved in reactions apparently proceeding to a physiologically significant extent in both directions. Brown (1953) was able to show that respiration as measured by oxygen absorption could proceed uninterruptedly in green plants in the light, together with photosynthesis. Benson & Calvin (1950) found that ^{14}C fixed during short periods of continuous photosynthesis by algae from $^{14}CO_2$ entered many of the phosphorylated intermediates also common to respiration, but was not found in certain compounds of the tricarboxylic acid cycle, such as citric and glutamic acids. Ryther (1956), using a marine flagellate uniformly labelled with ^{14}C, measured the loss of labelled carbon from cells when they were placed in a ^{12}C environment in the light and dark. After 24 hr, cells in the dark had lost about 20 % of their ^{14}C, but cells in the light had lost none. He concluded that respiratory carbon was used in preference to external carbon dioxide for photosynthesis, but was unable to come to any conclusions as to the form in which the carbon is returned to the photosynthetic cycle.

Weigl, Warrington & Calvin (1951), approaching closer to the problem of single or separate reservoirs, found that ^{14}C recently incorporated photosynthetically did not

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serve as a substrate for respiration in the light, but was evolved as $^{14}CO_2$ when the light was turned off after a short period of photosynthesis (40 min). After a sufficiently long period in the light (about 2 hr), there was no evolution of $^{14}CO_2$ in the subsequent period of darkness. They concluded that the evolution of $^{14}CO_2$ in the dark after a short light period was due to respiration of labelled photosynthetic intermediates, a process in some way inhibited in the light. However, after longer periods in the light, the cell having exhausted the external supply of carbon dioxide long before the light was extinguished, the incorporated ^{14}C would be found in stable storage materials. Respiration used other (unlabelled) storage materials as substrates, but whether these differed from photosynthetically formed reserve substances in chemical composition, or solely in physical location, could not be decided.

Direct information as to the identity of reservoirs is difficult to obtain, and in the present communication work is described which uses several indirect approaches. None of these alone is conclusive, but taken together they provide evidence strongly suggestive of a physical separation of metabolic pools of chemically identical substances participating in both respiration and photosynthesis in the *Chlorella*.

2. Methods

The green alga, *Chorella pyrenoidosa*, was grown for experimental purposes as described earlier (Bassham & Calvin, 1957). For feeding experiments with labelled glucose or acetate, 1 ml. aliquots of cell suspension in distilled water (containing 50 μl. of wet-packed cells) were pre-adapted for 30 min in the light or dark with a stream of air containing 1 % CO_2, and then incubated for 3 min in the light or dark with one or more of the following substrates: $NaH^{14}CO_3$ (20 μc; 1·35 μmoles); [^{14}C]glucose (33 μc; 0·75 μmoles), or an equal quantity of unlabelled glucose; $^{14}CH_3$. COONa (43 μc; 21 μmoles), or an equal quantity of unlabelled acetate. Except when $NaH^{14}CO_3$ was used as a substrate, the cell suspensions were flushed during the incubation period with air containing 1 % CO_2. At the end of the incubation period the cells were killed by the addition of 4 vol. of ethanol at room temperature. For experiments on the feeding of labelled acetate, the cells were starved before use by maintaining them for 24 hr in the dark in nutrient solution while they were flushed with air containing 4 % CO_2. The techniques used for the exposure of *Chlorella* to labelled substrates, both in the light and in the dark, are described by Moses & Calvin (1958*b*).

In "steady state" experiments, 50 ml. of cell suspension in a modified nutrient solution (containing 250 μl. of wet-packed cells) were placed in an apparatus modified from that described by Bassham, Shibata, Steenberg, Bourdon & Calvin (1956) and by Wilson & Calvin (1955). This was a closed system in which the atmosphere was recirculated and bubbled through the algal suspension which was exposed to light from two RS–P–2 spotlights on either side of a "lollipop"; the light intensity was just sufficient to saturate photosynthesis. Continuous automatic measurements were made of the O_2 and CO_2 concentrations and the radioactivity level in the circulating atmosphere, as well as of the pH and temperature of the algal suspension. The medium contained (g/l.): KNO_3, 0·75; $MgSO_4.7H_2O$, 0·10; KH_2PO_4, 0·05; plus the necessary trace elements; pH 6·6–6·9. After flushing the cell suspension with air containing 1·5 % CO_2 for 30 min, the system was closed and $^{14}CO_2$ released into the gas space (6 mc; final specific activity, 2 μc/μmole). Medium was added periodically to keep the cell suspension at a constant density as measured photoelectrically and a volume of cell suspension equal to that of the added medium was withdrawn. The rates of gas exchange during photosynthesis were 36–39 μmoles of O_2 evolved/min/ml. of wet-packed cells, and 34–39 μmoles of CO_2 utilized/min/ml. of wet-packed cells. Samples (1·5 ml.) of the suspension were taken 1·5 hr and 3 hr after the addition of $^{14}CO_2$, the cells killed with methanol at room temperature, and subsequently analyzed.

The procedures for the extraction of the cells, chromatography, radioautography, spot identifications, and radioactivity determinations, have been described elsewhere (Benson *et.al.*, 1950; Bassham & Calvin, 1957; Moses & Calvin, 1958*a*).

3. Results

Feeding experiments with labelled glucose and acetate

Chlorella was exposed, both in the light and dark, to three substrate combinations: (i) [^{14}C]glucose plus $^{12}CO_2$; (ii) [^{12}C]glucose plus $^{14}CO_2$; (iii) $^{14}CO_2$ alone. The patterns of ^{14}C distribution in most of the compounds in each extract are shown in Table 1. As the fixation of $^{14}CO_2$ in the dark, with and without glucose, was very low, and label appeared only in substances of the tricarboxylic acid cycle (Moses, Holm-Hansen & Calvin, 1958), these values have not been included in Table 1.

Table 1

Distribution of activity in various compounds after feeding Chlorella labelled glucose and carbon dioxide in different combinations in light and dark. For experimental details see text. 3 min exposure period to labelled substrates. The values given represent the percentage of the sum of the activities of all substances which appeared in each compound. About 92 % of the total soluble activity is accounted for.

Substrates	[^{14}C] glucose + $^{12}CO_2$	[^{14}C] glucose + $^{12}CO_2$	$^{14}CO_2$ + [^{12}C] glucose	$^{14}CO_2$
Light conditions	dark	light	light	light
Sugar diphosphates:				
glucose and sedoheptulose	0·2 %	1·1 %	1·8 %	1·8 %
fructose	0·04	0·8	1·0	0·6
ribulose	0·0	2·0	9·5	8·0
glyceric acid	0·0	0·2	0·6	0·6
glycollic acid	0·0	0·1	0·1	0·1
2-carboxy-4-keto-pentitol†	0·3	1·2	1·0	1·3
Sugar monophosphates:				
glucose	15·3	23·7	17·3	13·3
fructose	7·8	12·5	8·7	8·0
sedoheptulose	2·7	6·5	10·5	11·3
Pentose phosphate	0·7	1·9	1·1	1·5
Phosphoglyceric acid	6·7	6·7	18·9	26·1
Uridinediphosphoglucose	8·3	8·1	3·9	4·3
Malic acid	0·3	0·0	1·1	1·1
Glyceric acid	1·5	3·0	1·5	2·6
(Aspartic acid)‡	(5·4)	(19·2)	(4·7)	(5·2)
Glutamic acid	1·8	3·0	0·0	0·0
Alanine	10·0	9·6	17·6	17·0
Sucrose	44·2	19·2	5·2	2·4
Percentage of ribulose in diphosphates	0·0	36·9	68·0	64·4
Total ^{14}C fixed into soluble materials (dis/min/ml. cells $\times 10^{-6}$)	66·58	20·17	129·1	127·7

†Identity not definitely established.

‡Aspartic acid chromatographed together with a radioactive contaminant in the labelled glucose, and is thus not included in the total; it is also omitted from the total in columns 3 and 4 to provide data comparable with columns 1 and 2.

With acetate feeding, the following substrate combinations and light conditions were employed: (i) $^{14}CH_3$. COONa plus $^{12}CO_2$ in the dark; (ii), same as (i), but in the light; (iii) $^{12}CH_3$. COONa plus $^{14}CO_2$ in the light; (iv) $^{14}CO_2$ in the light. Table 2 shows the distribution of activity in various substances from each of these combinations.

TABLE 2

Distribution of activity in various compounds after feeding starved Chlorella labelled acetate and carbon dioxide in different combinations in the light and dark. For experimental details see text. 3 min exposure period to labelled substrates. The values given represent the percentage of the total soluble fixed ^{14}C which appeared in each compound.

Substrates	[2-^{14}C] acetate + $^{12}CO_2$	[2-^{14}C] acetate + $^{12}CO_2$	$^{14}CO_2$ + [^{12}C] acetate	$^{14}CO_2$
Light conditions	dark	light	light	light
Sugar diphosphates	0·0 %	0·0 %	21·5 %	24·0 %
Sugar monophosphates	0·0	1·2	13·0	16·0
Phosphoglyceric acid	0·0	0·0	2·9	4·3
Phosphoenolpyruvic acid	0·0	0·0	0·5	0·6
Uridinediphosphoglucose	0·0	0·0	2·1	4·4
Phosphoglycollic acid	0·0	0·0	1·5	1·4
Malic acid	2·2	3·5	4·8	7·0
Citric acid	12·1	22·5	3·9	0·1
Fumaric acid	0·0	0·0	1·2	1·1
Succinic acid	11·7	11·7	0·0	0·0
Glyceric acid	4·9	4·8	2·1	0·9
Lactic acid†	3·0	1·4	0·0	0·0
Glycollic acid	4·6	5·5	0·0	0·0
Sucrose	0·0	3·0	21·9	10·8
Aspartic acid	10·6	1·7	4·0	7·3
Glutamic acid	50·4	42·1	3·0	0·3
Serine plus glycine	0·0	0·6	11·6	13·8
Alanine	0·5	1·2	5·9	8·0
Glutamine	0·0	0·7	0·0	0·0
Total ^{14}C fixed into soluble materials (dis/min/ml. cells $\times 10^{-6}$)	21·56	23·36	390·3	266·9

†Identity not confirmed by chromatography.

Steady-state experiment

From a knowledge of the specific activity of the external $^{14}CO_2$ supplied to the cells, it is possible to determine the pool size of various compounds in the cells by assuming that these substances have the same specific radioactivity in their carbon atoms as the input $^{14}CO_2$. It is known that the photosynthetic intermediates rapidly acquire the same specific activity as the substrate carbon dioxide (Calvin & Massini, 1952). Thus, in the first sample, taken after 1·5 hr, the sizes of the photosynthetic reservoirs (and

of any other pools rapidly incorporating labelled carbon) can be determined from the amounts of ^{14}C in them. If the cells contain any other pools of these substances, not in rapid equilibrium with the photosynthetic pools, it would be expected that ^{14}C would enter these other reservoirs more slowly than those of the photosynthetic intermediates. For this reason the total pools of any such substances, measured on the basis of their ^{14}C content, should show a rise in absolute concentration until all the pools have the same specific activities as the incoming $^{14}CO_2$. Table 3 shows the apparent concentrations of several substances in the cells, as determined by the quantities of ^{14}C incorporated into them after 1·5 hr and 3 hr respectively. As already mentioned, a known volume of medium was added from time to time to the algal suspension, bringing the density (as measured by light absorption) back to the starting level, and an equal volume of cell suspension was removed, thus maintaining the suspension volume at a constant level. However, calculation of the algal growth during the course of the experiment from the rates of CO_2 uptake indicated that, on an increase in weight basis, there was a net increase in algal cell volume. A correction was made for this growth in calculating the apparent concentrations of photosynthetic pools. In calculating the increase in cell volume it was assumed that, (i) specific gravity of the average cell remained constant, (ii) each millimole of CO_2 incorporated resulted in an increase in dry weight of 30 mg (equivalent to CH_2O) and (iii) wet weight equaled five times dry weight. The validity of this calculation is indicated by the fact that rates of CO_2 uptake and O_2 evolution per unit volume of algal cells as determined by this calculation remained constant within 10 % during the course of the experiment. It will be noted that without such a correction for growth, the increase in calculated apparent concentrations of metabolic pools would have been greater than the increases shown in Table 3.

TABLE 3

Pool sizes of three compounds in Chlorella. Pool sizes given after continuous exposure of the alga to $^{14}CO_2$ in the light. Reservoir sizes determined on the basis of the incorporated ^{14}C into each compound; for explanation see text. Concentrations of each substance expressed as μmoles/ml. of wet-packed cells.

Exposure period to $^{14}CO_2$	Substance		
	Phosphoglyceric acid	Hexose and heptose monophosphates	Pentose monophosphates
1·5 hr	3·0	9·5	0·6
3·0 hr	6·4	11·4	0·7

4. Discussion

Glucose feeding

The pattern of ^{14}C incorporated from labelled glucose in the dark indicates that glucose is metabolized via glycolysis, since label appeared in several glycolytic intermediates (glucose and fructose monophosphates, phosphoglyceric acid, and

fructose diphosphate), and probably also by the pentose phosphate cycle as witnessed by the presence of ^{14}C in sedoheptulose and pentose phosphates. The one compound which plays a role in the photosynthetic carbon cycle but has no part in respiration, ribulose diphosphate, incorporated no radiocarbon from glucose in the dark. Previous studies (Bassham *et al.*, 1956) showed that the concentration of ribulose diphosphate in the photosynthetic pool goes to zero in the dark in *Scenedesmus*. Tricarboxylic acid cycle activity was also indicated by the presence of ^{14}C in malic and glutamic acids.

If the pools of photosynthetic and respiratory intermediates were not in equilibrium, no label should have been incorporated into ribulose diphosphate in the light unless $^{14}CO_2$ produced by oxidation of glucose were refixed photosynthetically or unless some glucose itself can reach and be incorporated into the photosynthetic pool. Experimentally, it was found that ribulose diphosphate was labelled in the light when [^{14}C]glucose was the substrate (Table 1), but it must be borne in mind that respiratory carbon is used in preference to an external source of this substance (Ryther, 1956).

A more definite conclusion regarding the separation of the pools may be derived from a consideration of the distribution patterns of ^{14}C from [^{14}C]glucose in the light and dark, compared with that from $^{14}CO_2$ in the light. The Q_{O_2} for oxygen evolved during photosynthesis is about 255 (36–39 μmoles of oxygen evolved/min/ml. of wet-packed cells; 1 ml. of wet-packed cells contained about 200 mg of dry cell material). Millbank (1957) has shown that the Q_{O_2} for the aerobic oxidation of glucose by *Chlorella vulgaris* is 12–13; hence photosynthesis proceeded about 20–21 times faster than respiration.

There was an increase in the percentage ^{14}C fixed from [^{14}C]glucose into the sum of the diphosphates in the light compared with the dark, and although label appeared in ribulose diphosphate, the percentage activity in fructose diphosphate also increased some twenty times. That fraction of ^{14}C in the diphosphates which appeared in ribulose diphosphate was much lower when [^{14}C]glucose was the substrate in the light (37 %), than the typical values for photosynthesis (64–68 %). From all these considerations it appears that the ^{14}C found in ribulose diphosphate arrived there by refixation of respiratory $^{14}CO_2$. Thus it is easily possible to account for the amount of labelled ribulose diphosphate (0·4/10 = 1/25) from labelled glucose compared with the ribulose diphosphate derived from labelled carbon dioxide, in terms of the reincorporation of labelled carbon dioxide derived from labelled carbon dioxide, and in terms of the reincorporation of labelled carbon dioxide derived from glucose. One would expect that, were the photosynthetic and respiratory pools in equilibrium, in the light virtually the whole of the distribution pattern of ^{14}C should have been typical of photosynthesis. Examination of the data in Table 1, however, shows that only a few compounds were affected by switching on the light when [^{14}C]glucose was the substrate. The most outstanding effect of light was the rise of the percentage of ^{14}C in the sugar monophosphates and the fall of that in sucrose. This may be accounted for by a relative lack of adenosine triphosphate necessary for the conversion of the sugar monophosphates to sucrose (via uridine diphosphoglucose), because of the rapid utilization of the nucleotide in photosynthesis. This might also explain the reduced total utilization of [^{14}C]glucose in the light, as the initial step of glucose incorporation also involves a phosphorylation. Although the *percentage* of ^{14}C found in uridine diphosphoglucose was the same in the light and dark (Table 1), the absolute amount was smaller by a factor of about 3·3 in the light in accordance with a corresponding reduction in the total ^{14}C incorporated in the light compared with the dark.

Perhaps most significantly, ^{14}C was found in glutamic acid from [^{14}C]glucose even in the light, but not from $^{14}CO_2$. Thus, glucose carbon, even though it passed through either glycolysis or the pentose phosphate cycle, and hence through compounds also present in the carbon reduction cycle, was able to enter glutamic acid in three minutes, whereas carbon fixed from $^{14}CO_2$ into these same intermediates by photosynthesis was not able to pass into glutamic acid. With photosynthesis proceeding some twenty times faster than respiration, the addition of unlabelled glucose to cells carrying on photosynthesis with $^{14}CO_2$ had, as may be expected, no significant influence on the ^{14}C uptake and distribution.

Acetate feeding

The picture of events obtained from the experiment with the feeding of labelled acetate is in some ways simpler to interpret. Very little label appeared in the phosphorylated compounds from [2-^{14}C]acetate in the light, and none in the dark. It therefore seems likely that only a little of the methyl-carbon of acetate was oxidized to carbon dioxide, and that the small labelling in phosphorylated compounds which was actually seen in the light was due to the refixation of this respiratory $^{14}CO_2$. In the tricarboxylic acid cycle, the methyl-carbon of acetate is not released as carbon dioxide during the first turn of the cycle. The pattern of labelling in the intermediates and amino acids of this cycle from [2-^{14}C]acetate in both light and dark indicates that very little acetate carbon travels all the way round the cycle as little or no activity was detected in fumaric and malic acids (Table 2).

The results with acetate confirm some of the conclusions from glucose studies. In particular, ^{14}C entered glutamic acid in the light from both substrates (but not from $^{14}CO_2$), demonstrating that the formation of this compound may proceed in the light. Other studies on the photosynthetic incorporation of tritium from tritium oxide (Moses & Calvin, 1958*b*), and the distribution of $^{14}CO_2$ in the presence of ammonia (Holm-Hansen, Nishida, Moses & Calvin, 1958), have also shown the formation of glutamic acid in the light, yet cells suspended in distilled water as in the present investigation formed none from $^{14}CO_2$ in 3 min, as was earlier found by Benson & Calvin (1950). Hence, carbon from photosynthetic intermediates is inhibited from entering tricarboxylic acid cycle substances by light in the absence of ammonia although other carbon in the same chemical compounds is not restricted in this way.

The incorporation of ^{14}C into aspartic acid from [2-^{14}C]acetate in the presence of $^{12}CO_2$ was suppressed in the light as compared with the dark. The incorporation of ^{14}C into aspartic acid from $^{14}CO_2$ in the light was suppressed by the presence of unlabelled acetate. These two effects suggest that a precursor of aspartic acid, malate or oxalacetate, may be synthesized both from acetate (via the glyoxalate cycle: see Kornberg & Krebs, 1957) and from photosynthetically incorporated CO_2 (via carboxylation of pyruvate or phosphoenolpyruvate) (Fig 1). In each case, the specific activity of the four-carbon acid would be reduced owing to the synthesis from the unlabelled substrate. Consequently, aspartic acid derived from the four-carbon precursor would have a lower specific activity and consequently a lower total activity, provided that its rate of formation were not increased owing to an increased concentration of its four-carbon acid precursor. The reason that the four-carbon acid precursor does not markedly increase in concentration in any case where acetate is present is that the rate of the condensation reaction between four-carbon acid and acetate is accelerated by acetate, as is seen in the increased labelling of citric acid and glutamic acid in light and $^{14}CO_2$ (in the presence of acetate). Further indication of the synthesis

of malate from acetate via the glyoxylate cycle is to be seen in the labelling of glycollic acid when [2-^{14}C]acetate was present.

Steady-state experiments

The evidence presented above for the existence of separate metabolic pools is further confirmed by the estimate of the pool sizes of three photosynthetic intermediates, and particularly phosophoglyceric acid, made on the basis of the rapidity with which they incorporate ^{14}C photosynthetically from $^{14}CO_2$. Calvin & Massini (1952) showed

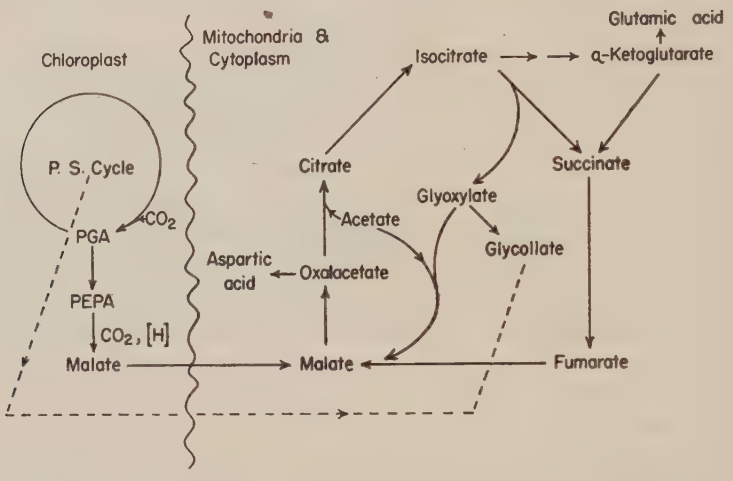

FIG. 1. Relationship between photosynthetic cycle, glyoxylate cycle and tricarboxylic cycle.

that in steady-state conditions the quantity of ^{14}C in phosphoglyceric acid rose rapidly for the first 5 min after the introduction of $^{14}CO_2$, and thereafter increased very slowly. This suggested that within five minutes photosynthetic phosphoglycerate had acquired the same specific activity as the external $^{14}CO_2$. The distribution of label in phosphoglyceric acid is known to be uniform after 5 min. However, the size of the labelled pool of this substance in algal cells increased two-fold between 1·5 hr and 3 hr after labelled carbon dioxide was added (Table 3). Thus, there appear to be one or more other reservoirs of phosphoglycerate not in rapid equilibrium with the pool involved in the carbon reduction cycle, and one of these other reservoirs may well be on a respiratory pathway.

Recent work by Tolbert (1958) has shown that phosphorylated intermediates are not released from chloroplasts (the site of photosynthetic activity) in the light. Investigations are currently in progress to see whether these various enzymatic activities are separately localized in different subcellular fractions.

REFERENCES

Bassham, J. A., Shibata, K., Steenberg, K., Bourdon, J. & Calvin, M. (1956). *J. Amer. Chem. Soc.* **78**, 4120.

Bassham, J. A. & Calvin, M. (1957). *Path of Carbon in Photosynthesis*. Englewood Cliffs, New Jersey: Prentice Hall, Inc.

Benson, A. A. & Calvin, M. (1950). *J. Exp. Bot.* **1**, 63.

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W. (1950). *J. Amer. Chem. Soc.* **72**, 1710.

Brown, A. H. (1953). *Amer. J. Bot.* **40**, 719.
Calvin, M. & Massini, P. (1952). *Experientia*, **8**, 455.
Holm-Hansen, O., Nishida, Y., Moses, V. & Calvin, M. (1958). *Effects of Mineral Salts on Short-Term Incorporation of Carbon Dioxide in Chlorella*. Submitted to *J. Exp. Bot.*
Kornberg, H. L. & Krebs, H. A. (1957). *Nature*, **179**, 988.
Millbank, J. W. (1957). *J. Exp. Bot.* **8**, 96.
Moses, V., Holm-Hansen, O. & Calvin, M. (1958). *The Non-Photosynthetic Fixation of Carbon Dioxide by Three Microorganisms*. Submitted to *J. Bact.*
Moses, V. & Calvin, M. (1958*a*). *Proc. Nat. Acad. Sci., Wash.* **44**, 260.
Moses, V. & Calvin, M. (1958*b*). *Photosynthesis Studies with Tritiated Water*. Submitted to *Biochim. biophys. Acta*.
Ryther, J. H. (1956). *Nature*, **178**, 861.
Tolbert, N. E. (1958). Brookhaven Biology Conference, No. 11.
Weigl, J. W., Warrington, P. M. & Calvin, M. (1951). *J. Amer. Chem. Soc.* **73**, 5058.
Wilson, A. T. & Calvin, M. (1955). *J. Amer. Chem. Soc.* **77**, 5948.

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An Electron Microscope Study of the Structure of Crystalline Ribonuclease

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Uranium and palladium shadowcast replicas have been used to study the surface structure of crystals of the monoclinic form II of the low molecular weight protein ribonuclease. $a \sin \beta$ has been deduced from the height of vertical steps on the (100) face; b and c have been obtained from the repeat distance between periodic shadows on the (100) face. Transmission microscopy has shown a periodic 18 Å line structure in thin frozen-dried crystal fragments. This structure is interpreted as being derived from a layered arrangement of protein molecules and water molecules in wet crystals which gives rise to alternate layers of high electron scattering power and low electron scattering power in the frozen-dried material. Moiré fringes have also been observed in thin crystal fragments. These fringes may be derived from rotation or misfit in the 18 Å periodic structure. The relationship of the electron microscope observations to proposed structures derived from X-ray diffraction studies is discussed.

No previous electron microscope studies of ribonuclease have been reported, although independent investigations by X-ray diffraction methods have been published by Carlisle & Scouloudi (1951), Carlisle, Scouloudi & Spier (1953), Magdoff & Crick (1955) and by Magdoff, Crick & Luzzatti (1956). The ribonuclease molecule is of comparatively low molecular weight, the commonly accepted value being 13,000. This low value indicates a small molecule and ribonuclease is therefore a promising subject for X-ray investigation. Conversely, the relatively small size of the molecule has meant that hitherto it has not been chosen for electron microscope study, since it is only comparatively recently that the resolving power available in commercial electron microscopes has been improved sufficiently to make such a study profitable. Results obtained in a small molecule of this kind, although less detailed than those from X-ray diffraction studies, are of particular value since they are direct observations and can be used to verify and supplement the X-ray results which are obtained by an indirect method.

In the preparation of crystalline material for electron microscope study several methods are available. The simplest, namely the direct examination of intact crystals which have been grown on the specimen mount or deposited there from a suspension, is only satisfactory for extremely thin crystals and is therefore of limited applicability. The various possibilities of this method have been reviewed previously (Dawson, 1953).

Thin section cutting methods have been employed by Farrant & Hodge (1956) in a study of ferritin crystals, a protein with a molecular weight of approximately 450,000. It is difficult at the present time to apply this method to the study of ribonuclease crystals since the molecule is some 35 times smaller than the ferritin molecule and correspondingly thin sections cannot yet be obtained.

The most successful method used for the study of protein crystals has been the method due to Wyckoff (1949), in which a so-called pseudo-replica of the crystal is

formed. It was this method which was found to give the best results in this investigation. However, in dealing with proteins of lower molecular weight than those originally studied by Wyckoff, some difficulties arise in the application of the method, and the techniques devised to circumvent these difficulties are outlined in the following section.

The crystals studied were of the monoclinic form II of the protein and were supplied by Dr. C. H. Carlisle.

Experimental

(a) *Specimen preparation*

In all the experimental techniques employed the crystalline material was first frozen dried as described for β-lactoglobulin by Dawson (1953). The material was supplied in the form of a suspension of micro-crystals in an alcohol/water mixture. A drop of this suspension was placed on a glass slide and the micro-crystals allowed to settle on the glass surface. On rapid freezing of the slide by placing it on top of a block cooled with liquid air, it was found that freezing took place from the bottom layer upwards; thus the protein crystals which had settled on the slide were first frozen to the surface and the protein in solution was driven upwards by an advancing front of ice, until finally this concentrated solution froze in a layer on top of the preparation. After careful vacuum drying at − 30°C and a pressure of less than 10^{-4} mm, it was found that the amorphous layer could be brushed off the surface to leave the crystalline material adhering to the slide. The methods used for examination of the frozen dried material were (i) carbon replication of pre-shadowed crystals, (ii) carbon replication and subsequent shadowing, and (iii) transmission microscopy of thin crystals.

(i) *Pre-shadowed carbon replicas*

The larger crystalline material on its supporting glass slide is shadowed with palladium in the normal way (Williams & Wyckoff, 1946) and then covered with a thin film of carbon (Bradley, 1954). The metal-carbon film can be stripped by water flotation, which also serves to extract the ribonuclease. The replica film is then mounted and examined in the usual manner. This method has the great advantage that the shadowing is carried out directly on the original material but it is not of great value in high resolution work owing to the granularity of palladium shadowcast layers. Other shadowcasting metals which give a better result do not allow stripping of the replica by water flotation.

(ii) *Post-shadowed carbon replicas*

Here the carbon film is evaporated directly on to the crystals and stripped by the method described above. By placing specimen grids on top of the floating film and superimposing a piece of absorbent paper, it is possible to lift the film from the water surface and to reverse it so that the replica is uppermost on the mount. The surface can then be shadowcast with uranium, which is found to be more suitable for high resolution microscopy.

A modification introduced here was to obtain smaller crystals from an amyl acetate suspension of some of the finer surface material removed after freeze drying. Drops of this suspension are placed on a glass slide and the resultant material treated as before.

It is found that sometimes the crystalline material is imperfectly extracted from the replica and transmission microscopy is occasionally possible on such residual material.

(iii) *Transmission microscopy*

The results of transmission microscopy of the residual material on replicas can be confirmed by direct transmission microscopy of thin crystals mounted on formvar-covered specimen grids. Such specimens can be obtained by evaporation on the formvar-covered mounts of drops of the amyl acetate suspension previously mentioned. It is found that the best results are obtained by evaporation of freshly prepared suspensions on mounts supported in a metal tray, which is placed on a block cooled with liquid air immediately after deposition of the suspension on the film. A standard freeze drying cycle is then carried out.

(b) *Electron microscopy*

(i) *Replicas*

In general, electron microscopy of the replicas was dealt with as previously described by Dawson (1951, 1953).

(ii) *High resolution microscopy*

The Siemens Elmiskop I provides two alternative modes of illumination: with a single condenser (approximating to the conditions in the Philips microscope used in the previously described work on β-lactoglobulin (Dawson, 1951)), or with a double condenser. The latter produces a very fine illuminating pencil which effectively limits irradiation of the specimen to the area under examination. The double condenser system increases resolution since it produces an illuminating pencil in which the rays are almost parallel, thus allowing optimum performance of the objective lens.

Thus, in high resolution microscopy, the use of the double condenser system is to be preferred. The main drawback is the rapid accumulation of a contaminating layer on the small area of the specimen illuminated by the double condenser. Structure visible on the first of two successive plates of the same field is frequently obliterated on the second.

The double condenser system is not very suitable for specimens shadowcast with the metals usually employed. Granulation is invariably accentuated. However, the uranium-shadowed replicas were found to be suitable for examination using double condenser illumination: this proved convenient in that it also allowed examination of residual crystals on such replicas by transmission under the condition most favourable for high resolution.

Results

(a) *Pre-shadowed carbon replicas*

These were used to measure the height of vertical growth steps on the (100) face from the lengths of the shadows cast by them. The step height is 28 ± 4 Å (mean of observations made on 53 crystals) which gives a value for $a \sin \beta$. Plate I (a) shows a micrograph of such a replica. (The measure of variability given after the $\pm$ signs in this section is the standard deviation of individual measurements from the mean.)

(b) *Post-shadowed carbon replicas*

These were used for high resolution studies with the Siemens Elmiskop I. We have observed periodic shadows on the (100) face parallel to the b-axis, similar to those described for β-lactoglobulin by Dawson (1951). Measurement of the spacing of these on 15 crystals gives a value of 49 ± 3 Å for c. On one crystal, measurement of the

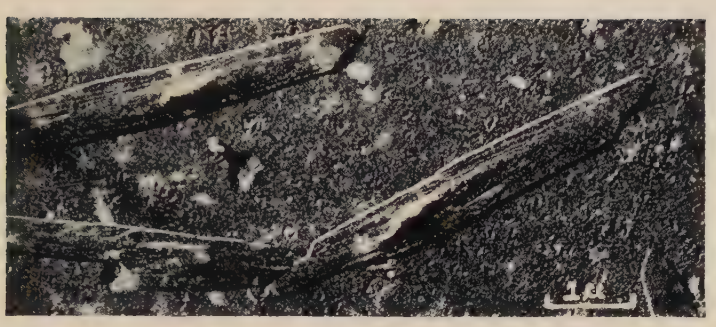

PLATE I(a). Electron micrograph of palladium pre-shadowed carbon replica of ribonuclease crystals. The heights of vertical steps on the (100) face shown give a value for $a \sin \beta$.

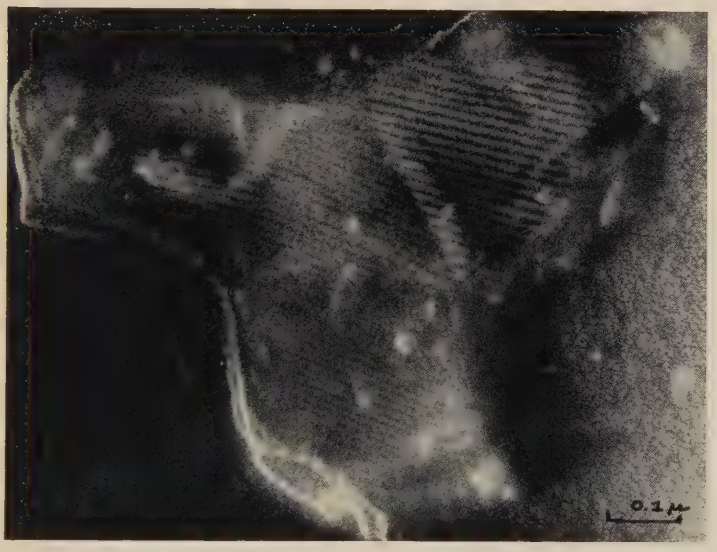

PLATE I(b). Electron micrograph of uranium-shadowed carbon replica of ribonuclease crystal. The residual crystalline material shows periodic structure unrelated to the direction of shadowing. Negative print.

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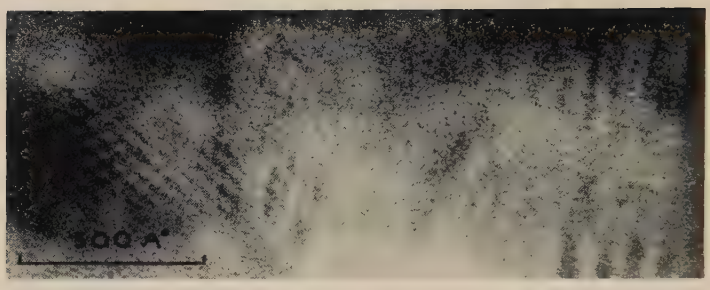

PLATE II(a). Transmission electron micrograph of frozen dried ribonuclease crystal showing moiré fringes of varying dimensions.

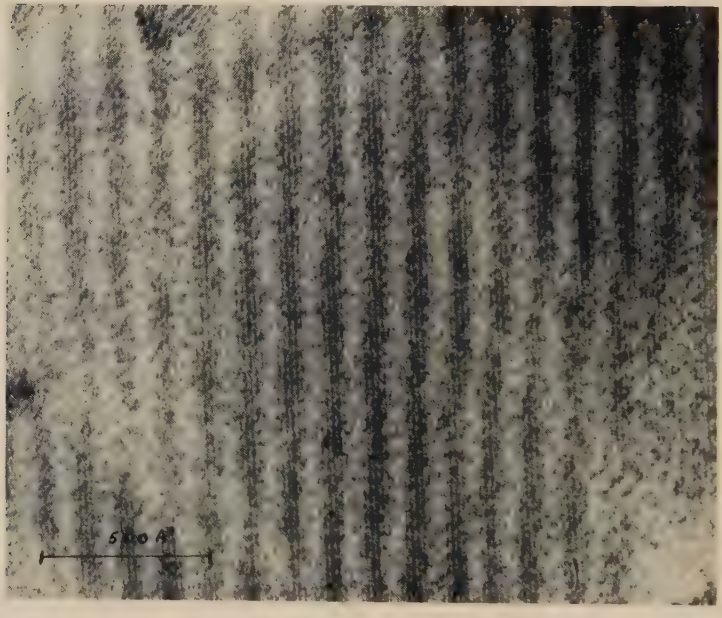

PLATE II(b). Electron micrograph of small area of crystal shown in Plate I(b), showing periodic structure. The fine spacing is 18 Å, the coarse spacing 127 Å. Positive print.

periodicity of shadows parallel to the *c*-axis gives a value of 32 Å for *b*. The directions of the *b* and *c* axes were identified by comparison with the descriptions and diagrams of the crystal habit given by Carlisle & Scouloudi (1951) and by Magdoff & Crick (1955).

(c) *Transmission microscopy*

i) *Residual crystals on replicas*

Plate I (b) shows a negative micrograph of a residual crystal on a post-shadowed carbon replica. This micrograph was at an original magnification of 40,000 and has been enlarged to 95,000. Structure is visible over a wide area of the crystal. This structure is rather complex in form, exhibiting several distinct orientations, and has only been observed on this one crystal. There are, first, a series of broad black bands which have a repeat period varying between 120 Å and 140 Å over the crystal surface. There are also series of finer lines (shown more clearly on a positive micrograph at a higher magnification of 450,000 (Plate II (b)) almost, but not quite, parallel to the broad bands. These are observed at various sites on the crystal but the repeat spacing is always constant at 18 Å. The angle between the two series of bands cannot be accurately measured but is approximately 2° where the broad band spacing is 120 Å. It appears to be slightly less than 2° where the broad band spacing is 140 Å. The fine lines occur right across the area shown in Plate II (b) but the alternating intensities produced by the broader bands make them more readily visible at some part of the surface than at others.

Plate I (b) shows quite clearly that the band structures are unrelated to the direction of shadowing since their predominant direction is parallel to the shadowing direction: they are, in fact, due to real structure viewed in transmission. On the crystal shown here the fine bands have been resolved at some sites into two finer lines whose spacing (measured from one line to a corresponding point on the next) is 7–8 Å.

ii) *Direct transmission microscopy*

Although we have only encountered the fine band and related broad band spacing on the crystal, the fine bands have also been observed by direct transmission microscopy of frozen dried crystals as described in para. (a)(iii) of the Experimental section. Since such crystals are not so uniformly thin as the residual material on a replica, the structure has not been observed so clearly or over such a wide area of these specimens and it is not easy to determine whether the broad bands are present or not. It is, however, quite clear that ribonuclease crystals can show fine structure and broad fringes in the electron microscope. This confirms that the crystal of Plate I (a) is ribonuclease and not a fragment of graphite thrown onto the specimen during the carbon evaporation cycle.

Plate II (a) shows a micrograph of part of a frozen dried crystal examined by transmission microscopy. The fine spacing is not clearly visible but there is a well developed pattern of broader fringes (periodicity 50 Å) over part of the area shown.

Measurements on such crystals suggest a value of 16 $\pm$ 1 Å for the fine spacing (mean of 50 measurements on 12 crystals). This appears to be slightly lower than the value obtained from the crystal of Plate II (b).

Discussion

(a) *Unit cell dimensions and the 18 Å spacing*

The micrographs illustrated in Plates I (b), II (a) and (b) do not give a clear correlation between the unit cell as determined by X-ray analysis and the axial parameters

in crystal fragments. Other micrographs, such as Plate I (a) which we have obtained from replicas of crystals with well-defined faces, give values which agree well with the known values. The values obtained by Carlisle & Scouloudi (1951) and by Magdoff & Crick (1955) for the unit cell of the monoclinic form II are as follows:

	Carlisle & Scouloudi		*Magdoff & Crick*	
	Air-dry	*Moist*	*Air-dry*	*Moist*
a	29·1 Å	30·9 Å	30·4 Å	30·5 Å
b	30·1 Å	38·8 Å	33·4 Å	38·3 Å
c	51·0 Å	54·1 Å	53·0 Å	53·3 Å
β	114°	106°	113°	106°
$a \sin \beta$	28·0 Å	29·3 Å	26·4 Å	29·3 Å

The space group is $P2_1$ and the unit cell contains two molecules.

Our values quoted above, $a \sin \beta = 28 \pm 4$ Å, $b = 32$ Å and $c = 49 \pm 3$ Å, would appear to be consistent with these results. The results for air-dry crystals in the above table suggest that those studied by Carlisle & Scouloudi contained less moisture than those studied by Magdoff & Crick. Carlisle & Scouloudi estimate that their air-dry crystals contain about 14% by weight of water. Frozen dried crystals should contain less moisture than this but, since few measurements have been made on frozen dried crystals, the dehydration of protein crystals beyond the air-dried stage is as yet little understood.

The electron microscope measurements of unit cell parameters are less accurate than X-ray diffraction measurements; but within the limits of accuracy of the method used, the air-dried and electron microscope measurements are in reasonably good agreement. The most interesting feature of the X-ray results, in view of the observed strong periodicity in the electron micrographs, is the marked shrinkage which occurs in the b-axial dimension on air drying moist crystals. This shrinkage and the observed periodicity would suggest that the water in the lattice is accommodated in intermolecular layers parallel to the c-axis. The 18 Å spacing is approximately $b/2$ and since there are two molecules in the unit cell, this 18 Å spacing is consistent with an alignment of strongly scattering material in each molecule parallel to the c-axis. Since there are no heavy atoms in the molecule this periodic structure may relate to an alignment of amino acid residues parallel to the c-axial direction.

The value of 16 Å is closer to the value of $b/2$ expected for a dry crystal than the 18 Å value: this may due to the reduced possibility of water uptake by the crystals prepared for transmission microscopy.

(b) *The moiré fringes*

The moiré fringes seen in ribonuclease are the first patterns of this kind to be observed in protein crystals. Such fringes have been reported in other and simpler materials, notably graphite (Mitsuishi, Nagasaki & Uyeda, 1951), iron oxide (Hillier, 1954), molybdenum trioxide (Dowell, Farrant & Rees, 1956) and superimposed thin metal foils (Pashley, Menter & Bassett, 1957; Bassett, Menter & Pashley, 1958). In these cases it is easy to see how the fringes are derived from the primary lattice array by twist of successive layers of molecules through stacking faults, or by misfit of successive parallel layers in a two-component system. Our experiments with ribonuclease suggest that in this protein the appearance of fringes is related to the residual hydration of the crystal fragment and this in turn would imply that the moiré pattern is caused by variation in the lattice spacing, rather than by layer

rotation. No attempt has yet been made to control the hydration of the specimen, however, and until this is done and a considerable number of observations made, no definite answer to this problem can be given.

In the other examples cited above, the moiré fringes are all derived from lattice parameters of less than 10 Å and the crystals themselves exhibit a high degree of order in their structure. Although the ribonuclease crystal is much more complex, it gives both a moiré pattern and measurable X-ray reflections out to values of 1·25 Å (Carlisle & Scouloudi, 1951). Both these observations favour a highly ordered structure for the ribonuclease molecule.

(c) *Relation of present results to molecular models derived from X-ray diffraction*

The molecular models derived for ribonuclease from X-ray diffraction are not as yet fully developed: this renders it impossible to make any valuable interpretation of the present results on the basis of these models. However, there is still some interest in examining the suggested models in their present state for features which could possibly account for the experimental observations.

Carlisle *et al.*, (1953) suggest that there are six polypeptide chains in the molecule aligned parallel to the *c*-axis. The molecular packing in the crystal is such that on viewing the crystal down the $a \sin \beta$ axis, three of the six polypeptide chains in each of the two molecules in the unit cell are seen superposed. Thus there will be planes of high electron density in the (100) projection parallel to the *c*-axis and separated by a distance of $b/2$, which are conceivably related to the 18 Å spacing.

It is tempting to interpret the unconfirmed 7–8 Å spacing as being due to the spacing between the superposed trio of pencils in each molecule and a superposed pair of two of the remaining pencils in the molecule. The *c*-axial electron density map (Carlisle *et al.*, 1953) does show such a pair at a distance of just under 8 Å from the trio.

On the other hand, Magdoff *et al.*, (1956) claim that there is no evidence for parallel polypeptide chains in ribonuclease. They doubt if there are any definite polypeptide chain directions in the molecule at all but, if any do exist, then there are two chains in the *ac* plane and three in general directions outside this plane. The two in the *ac* plane could, of course, give an 18 Å spacing in a similar manner to the Carlisle model. The model in its present state offers no possible explanation for the 7–8 Å spacing. However, this is scarcely significant in view of both the present stage of development of the model and the lack of confirmation of our unique observation of this spacing.

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REFERENCES

Bassett, G. A., Menter, J. W. & Pashley, D. W. (1958). *Proc. Roy. Soc.* A, **246**, 345.
Bradley, D. E. (1954). *J. Inst. Met.* **83**, 35.
Carlisle, C. H. & Scouloudi, H. (1951). *Proc. Roy. Soc.* A, **207**, 496.
Carlisle, C. H., Scouloudi, H. & Spier, M. (1953). *Proc. Roy. Soc.* B, **141**, 85.
Dawson, I. M. (1951). *Nature*, **168**, 241.
Dawson, I. M. (1953). *Brit. J. Appl. Phys.* **4**, 177.
Dowell, W. C. T., Farrant, J. L. & Rees, A. L. G. (1956). *Proc. Int. Conf. Electron Microscopy*, London, 1954, p. 279. London: Royal Microscopical Society.
Farrant, J. L. & Hodge, A. J. (1956). *ibid.* p. 118.
Fillier, J. (1954). Nat. Bur. Stand. Circ. no. 527, *Electron Physics*, p. 413.
Magdoff, B. S. & Crick, F. H. C. (1955). *Acta Cryst.* **8**, 461.

Magdoff, B. S., Crick, F. H. C. & Luzzatti, V. (1956). *Acta Cryst.* **9**, 156.
Mitsuishi, T., Nagasaki, H. & Uyeda, R. (1951). *Proc. Imp. Acad. Japan*, **27**, 86.
Pashley, D. W., Menter, J. W. & Bassett, G. A. (1957). *Nature*, **179**, 752.
Williams, R. C. & Wyckoff, R. W. G. (1946). *J. Appl. Phys.* **17**, 23.
Wyckoff, R. W. G. (1949) *Electron Microscopy*, p. 72. New York: Interscience.

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Purification and Properties of Bacteriophage ϕX174†‡

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Procedures are described for the isolation in pure form of the bacterial virus ϕX174. This virus is shown to have a particle weight of $6{\cdot}2 \times 10^6$ and to contain 25 % by weight of DNA.

At neutral pH, the purified virus forms a discrete aggregate, probably a tetramer. This aggregate dissociates either upon dilution or at alkaline pH.

Lysates produced by ϕX174 contain a second particle, antigenically related to the virus, but not infective and of lower sedimentation rate and lesser DNA content.

The DNA of ϕX174 appears to have an unusual structure in that it reacts with formaldehyde (even before extraction from the virus) and in that the atomic efficiency of inactivation of the virus by decay of incorporated phosphorus-32, calculated by combining our data and that of Tessman, is 1·0.

The results of ultrafiltration and radiosensitivity experiments have indicated the existence of a class of very small bacterial viruses including notably S13 (Lea & Salaman, 1946; Zahler, 1958) and ϕX174 (Sertic & Bulgakov, 1935; Sertic, 1937*a* and *b*; Bulgakov & Bonét-Maury, 1944; Bonét-Maury & Bulgakov 1944; Frilley, Bulgakov & Bonét-Maury, 1944; Lépine, Bonét-Maury, Bulgakov & Giuntine, 1944). However, no physical or chemical studies have previously been made upon a purified preparation of a member of this class.

Interest in this class of bacteriophage stems, in part, from the potential value of comparisons of the physico-chemical and biological properties of these viruses with the better known properties of the more widely studied class of larger bacteriophages such as T2, and, in part, from their possible utility as a source of a molecularly homogeneous preparation of deoxyribosenucleic acid (DNA).

This paper is concerned with the isolation of pure preparations of ϕX174 and the physico-chemical characterization of this virus. The following paper will discuss some unusual properties of the DNA of ϕX174.

Material and Methods

Growth of the virus

Bacteriophage ϕX174 was obtained from Dr. N. Bulgakov. The virus is grown on *E. coli* strain C (BTCC no. 122). For mass cultures, 2 l. of the glycerol-casamino acid medium described by Fraser & Jerrel (1953) are inoculated at 37°C with 60 ml. of overnight cultures of C grown in the same medium. The cultures are then grown at 37°C with vigorous aeration. When the bacterial concentration has reached 10^9 per ml. (as checked with a Petroff-Hausser counter), virus is added to provide a final concentration of 10^{10} per ml. Foaming at lysis, which occurs about 45 minutes after inoculation, is controlled by

† This work was initiated while the author was on the staff of the Physics Department of Iowa State College.

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addition of Dow-Corning anti-foam B. After the initial lysis, aeration of the cultures is continued until 5 hr after addition of the bacteriophage inoculum. The lysates are then stored in the cold room until 20 l. are accumulated for purification. The titer of such lysates varies from 2 to 8 $\times$ 10^{11} per ml.

Assay of the virus

Assay of the virus is performed by the usual agar layer method (Adams, 1950), upon overnight cultures of C, grown in a medium containing 10 g/l. Bacto-tryptone, 5 g/l. KCl, and 5 $\times$ 10^{-4} M-calcium chloride. "Bottom" agar is made up with 10 g agar, 10 g tryptone, 2·5 g NaCl, 2·5 g KCl, and 6 ml. 1 N-NaOH per liter. After autoclaving, 1 ml. of sterile 1 M-$CaCl_2$ is added per liter. "Top" agar is made up with 0·5 g NaCl, 0·8 g agar and 1 g tryptone per 100 ml.

Purification of the virus

Lysates should not be allowed to stand more than one or two weeks in the cold room as loss of titer is observed. In our laboratory 16 or 20 l. of lysate are grown per week, and these are worked up as a batch.

A liter of lysate is poured into each of 20 2-liter beakers. Ammonium sulphate is added to 40 % saturation (280 g per liter) and 25 ml. of 0·1 M-versene pH 7·0 are added per liter. After thorough stirring, the precipitate is allowed to settle out for 48 hr in the cold room. After settling, about 700 ml. can be siphoned from each beaker. The sediments are pooled and allowed to settle a second time for 48 hr. After removing about 700 ml. of supernatant from each beaker by siphon, the pooled sediments (about 1500–2000 ml.) are spun out in the Sorvall SS2 centrifuge at 38,000 *g* for 30 minutes. The supernatants, which contain less than 10 % of the initial virus titer, are discarded.

The pooled sediments are resuspended by stirring for 2 hr on a magnetic stirrer in 100 ml. borate-versene.† To elute the virus from the sediment, the pH is raised to 9·7 by addition of 3 N-LiOH at the pH meter, and the suspension stirred an additional 8 hr. The pH is checked periodically and raised again to pH 9·7 if necessary. The suspension is then centrifuged at 7000 *g* for 30 min. The supernatant is immediately dialyzed against borate-versene.

The sediment is resuspended in 50 ml. borate-versene, stirred for 2 hr, brought to pH 9·7 with 3 N-LiOH, stirred an additional 8 hr and spun as above. Elutions of the sediment with decreasing volumes of borate-versene at pH 9·7 are repeated, until the sediment contains less than 5 % of the initial virus titer. Either 4 or 5 elutions may be required. The dialyzed supernatants are pooled and should contain about 40 % of the initial titer (about one-half of the initial titer is irreversibly lost during these procedures).

The pooled dialyzed supernatants are then spun at 7000 *g* for 30 min and any sediment is discarded. Ammonium sulphate is again added to 40 % saturation and the preparation is allowed to settle for 24 hr in the cold room. The suspension is then spun at 38,000 *g* for 30 min and the supernatant is discarded. The sediment is easily resuspended in 25–30 ml. of borate-versene by gently stirring for 2 hr. It is then dialyzed for 24 hr in the cold room against GSV solution.‡

After dialysis the virus suspension is spun at 6000 *g* for 20 min. The supernatant is stored. The sediment is resuspended in 10 ml. of borate, stirred for 2 hr and spun at 2000 *g* for 25 min. The supernatant is pooled with the previous supernatant and the sediment is discarded. The pooled supernatant should now contain about 20 % of the initial virus titer.

Within 48 hr the virus is spun out in the O rotor of the model E ultracentrifuge at 42,040 rev/min for 25 min.§ The supernatant is discarded, the sediment in each tube is covered with 1 to 1·2 ml. borate and allowed to stand 24 hr or longer in the cold room. The pellets are then resuspended by very gentle agitation and stirring for 2 hr in about

† The borate buffer referred to in this paper is a saturated (at 4°C) solution of sodium tetraborate, of pH 9·1. Borate-versene is 40 parts saturated borate and one part 0·1 M-versene pH 7·0.

‡ GSV is a solution containing, in final concentration, 0·2 M-NaCl, 0·01 M-versene, and 0·1 M-glycine, adjusted to pH 10·3 with NaOH.

§ If more than 48 hr must elapse before centrifugation, the virus is dialyzed back into borate in which it is much more stable than it is at pH 10·3.

PLATE I. Electron micrograph of ϕX174. The polystyrene spheres are 132 mμ in diameter.

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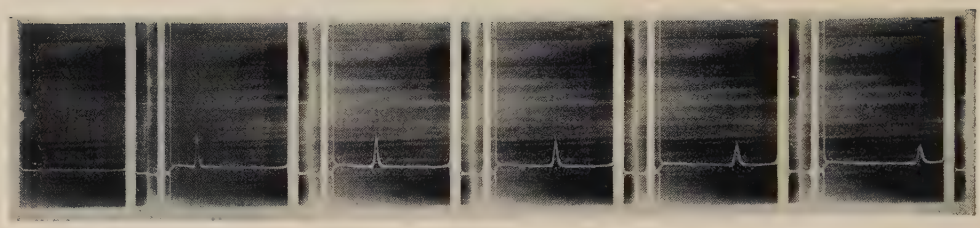

PLATE II (a). Ultracentrifuge sedimentation pattern of purified ϕX174. Pictures taken every 4 min at 31,410 rev/min.

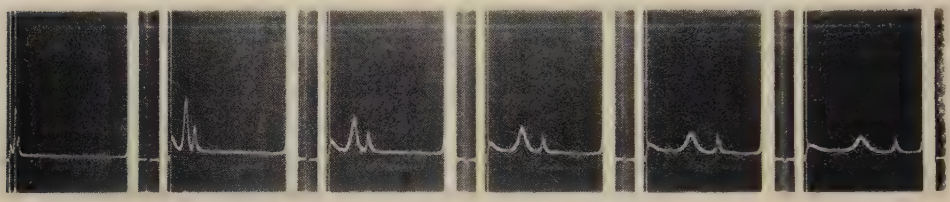

PLATE II (b). Ultracentrifuge sedimentation pattern of ϕX174 preparation before density gradient fractionation. The larger, slow component is the 70*S* fraction. Pictures taken every 4 min at 31,410 rev/min.

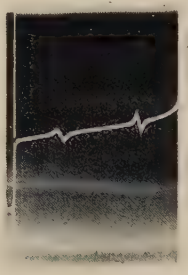

PLATE II (c). Schlieren pattern showing the 70*S* particles (left) and the ϕX174 particles (right) banded in a CsCl density gradient. The mean density is 1·36, the speed is 44,770 rev/min, and equilibrium was reached in 16 hr.

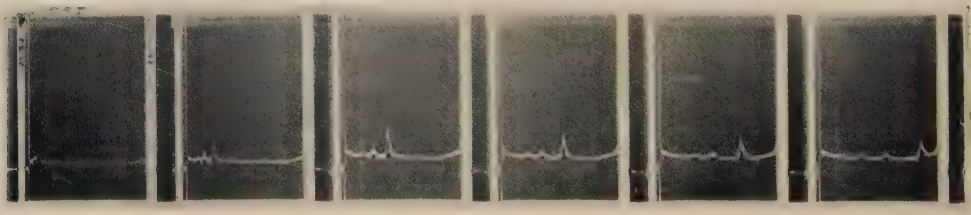

PLATE II (d). Ultracentrifuge sedimentation pattern of purified ϕX174 at pH 6·4. The leading component is the aggregate of $S = 155$. Pictures taken every 4 min at 31,410 rev/min.

10 ml. of borate. The suspension is then spun at 2000 *g* for 25 min. The supernatant is carefully decanted and saved; the sediment is resuspended in 5 ml. of borate and titered. If a significant titer is found, it is spun as above and the supernatant pooled with the previous supernatant.

The virus is again spun out in the O rotor as before. The sediment is resuspended as before in about 5 ml. of borate and given a final low speed spin. The sediment is discarded.

The virus concentration is now 1 to 3 $\times$ 10^{14} per ml. and will account for 10 to 20 % of the initial titer. The final step in purification of the virus is isolation of the virus band from a density gradient ultracentrifuge tube.

To 10 g of the virus suspension, purified as above, with a titer greater than 10^{14} per ml., are added 5·40 g of rubidium chloride (98·4 % rubidium chloride, obtained from American Potash and Chemical Corp., 3030 W. Sixth St., Los Angeles 54, Calif.). After solution of the rubidium chloride and thorough mixing, the density of the solution should be 1·33 $\pm$ 0·01. The 13 ml. of solution are divided equally into the three lusteroid tubes of a thermister-equipped, blackened SW39 swinging bucket rotor from the Model E ultracentrifuge. The suspension is then spun for 20 hr at 37,020 rev/min at 2·5°C. The virus bands at a density of 1·40 in the density gradient (Meselson, Stahl, & Vinograd, 1957) thus produced.

Upon completion of the run, the speed is lowered gradually to avoid convection and the lusteroid tubes are carefully removed. Three bands are observed in each tube: a light-brown, jelly-like pellicle at the surface; a broad, white diffusely scattering band, centered about 1 cm or less from the top surface; and a narrow, dense white band about 1 cm from the bottom of the tube. The pellicle is carefully removed with a small spatula and discarded. The diffuse band—which is the "70*S*" material (*vide infra*), is carefully removed with a syringe and similarly all liquid to within about 2 mm above the virus band is removed. The walls of the tube above the remaining liquid are wiped clean and the cell is gently shaken to break up the virus layer. The contents of the three cells are now pooled, the cells are rinsed with borate, and the washings are added to the suspension. After stirring on a magnetic stirrer with a glass-enclosed iron "flea" for 30 min, the virus suspension is dialyzed against 3 changes of borate. The final volume of phage is about 4 ml., of titer 2 to 5 $\times$ 10^{14} per ml. 75 to 100 % of the virus titer is recovered from the density gradient ultracentrifugation. At this stage, 25 to 50 % of the virus particles present are plaque-formers.

Properties of the Virus

Physical properties

An ultracentrifuge pattern of the purified virus in borate buffer at pH 9 is shown in Plate II (a). The sedimentation constant, $S_{20,w}$, is 114 *S*.

An electron micrograph of ϕX174 is shown in Plate I.† The virus appears to be a polyhedral object about 30 mμ in diameter.

A light scattering envelope of the virus is shown in Fig. 1. The refractive increment of the virus was measured to be 0·185 $\times$ 10^{-3}/mg/ml. on a Phoenix differential refractometer. (Dry weight was determined from an aliquot desiccated at 30°C, and then dried *in vacuo* over P_2O_5 successively at 55°C and 110°C.) From the intercept the particle weight is computed to be 6·2 $\times$ 10^6. The dissymetry, 1·04, would, for a spherical model, indicate a diameter of about 29 mμ (Doty & Steiner, 1950).

An ultraviolet absorption spectrum of the purified virus in borate buffer is shown in Fig. 2.‡ The specific absorption at 260 mμ is 8·14/mg/ml. For purified virus the

† This picture was taken at the Physics Department of Iowa State College by Mr. Robert Woodley, with the assistance of Dr. Percy Carr and the author.

‡ This spectrum was obtained on a Beckman DK2 spectrophotometer. With a suspension of large particles, such as this virus, scattering contributes significantly to the observed absorption and slightly different spectra will be obtained on other spectrophotometers with different geometries.

ratio of absorption at 260 mμ to that at 280 mμ is 1·53 and the absorption minimum is at 240 mμ. These two characteristics are sensitive indicators of impurities.

Chemical analysis

The virus contains 29·5 mg phosphorus per gram. The DNA content of the virus is 25·5 % by both the diphenylamine test (Dische, 1930) and the *p*-nitrophenylhydrazine test of Webb & Levy (1955). (Thymus DNA of known phosphorus content was used as a standard.) This amount of DNA is in good accord with that computed

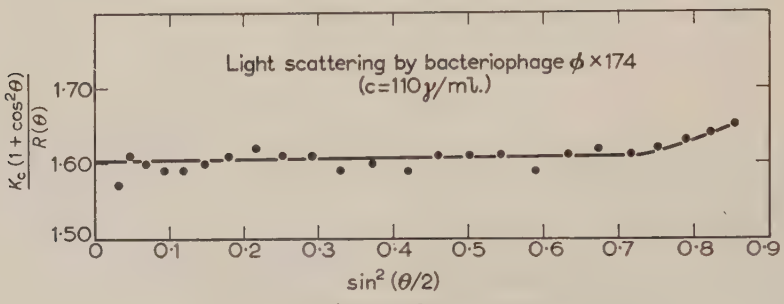

Fig. 1. Light scattering envelope of purified ϕX174.

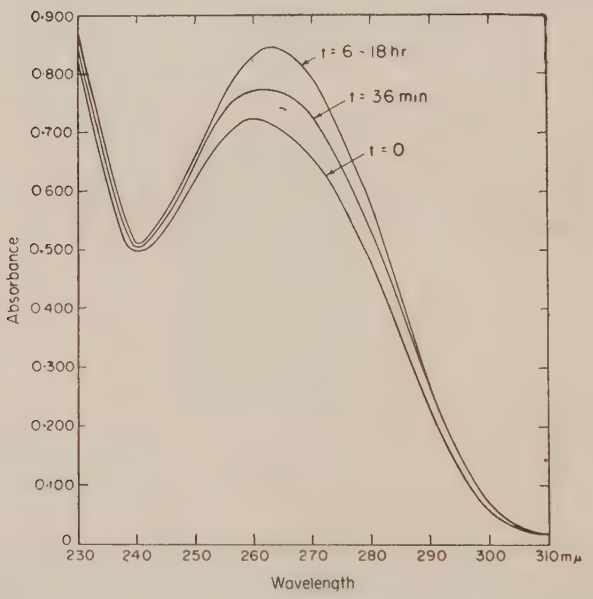

Fig. 2. Change in the ultraviolet absorption spectrum of ϕX174 upon the addition of formaldehyde at 37°C. Virus concentration = 89 μg/ml.; formaldehyde concentration = 1·8 %.

from the measured density, 1·40, observed in the density gradient ultracentrifuge runs. The DNA thus accounts for 80 % of the phosphorus. No RNA could be detected (RNA content certainly less than 0·5 % of virus weight) by either the Ogur & Rosen (1950) procedure or by the phloroglucinol procedure (von Euler & Hahn, 1946). The chemical nature of the residual phosphorus is unknown.

Upon treatment with formaldehyde the ultraviolet absorption spectrum of the virus changes (Fig. 2). This shift is identical with the change in absorption of a solution of RNA (Fraenkel-Conrat, 1954; Staehelin, 1958) or of denatured DNA (Hoard, 1957) when treated with formaldehyde and represents a reaction of the

reagent with free amino groups of purines and pyrimidines. This reaction is not observed for ordinary DNA in which the amino groups are protected by hydrogen bond linkage. Observation of this reaction with ϕX174 indicates that many of the amino groups of the DNA of this virus are not hydrogen-bonded as in the conventional two-stranded DNA (Watson & Crick, 1953). Other observations of the unusual nature of the DNA of ϕX174 will be reported in a subsequent paper.

70*S* *component*

An ultracentrifuge pattern of a virus preparation just before the density gradient step of purification is shown in Plate II (b). A second and larger component of $S = 70$ is observed, in addition to the viral component of $S = 114$. The 70*S* component, which has only been observed in virus lysates, is antigenically related to the virus but is not infective. It has 3·5 to 4·5 % DNA content by weight and bands at a density of 1·32. It was impossible to separate this component from the virus by either ammonium sulphate fractionation or by electrophoresis at various pH, and only the use of the density gradient method has permitted resolution of these two particles (Plate II (c)). Light scattering studies indicate the 70*S* component is not homogeneous in size or shape. DNA in the 70*S* component is protected from DNAse action. When isolated, the DNA of the 70*S* component shows a diffuse boundary in the ultracentrifuge with a sedimentation constant of about 9*S*.

Aggregation

At slightly acid or neutral pH in concentrations of 1 mg/ml. or greater, purified ϕX174 reversibly forms a discrete aggregate which has a sedimentation constant of 155*S* (Plate II(d)). Upon dilution or increase of pH, this aggregate dissociates to the monomeric form ($S = 114$). Because of this dissociation, light scattering measurements upon the aggregate could not be extrapolated to zero concentration in the usual way. From such light scattering data, an approximate weight of 25×10^6 can be estimated, suggesting that the aggregate is a tetramer.

Discussion

With a particle weight of $6{\cdot}2 \times 10^6$, ϕX174 is one-fortieth the mass of the T2 bacteriophage and is in the same size range as the smallest plant (Markham & Smith, 1954) and animal (Schwerdt, 1957) viruses. However, in contrast to the small plant or animal viruses which contain RNA, ϕX174 contains DNA. The DNA content of a ϕX174 particle is less than 2 % of the DNA content of a T2 particle (Hershey, Dixon & Chase, 1953), suggesting that ϕX174 must be both less complex and more dependent upon the functional metabolism of its host, than is T2.

The 70*S* component appears to be externally nearly indistinguishable from the virus. It is, however, non-infective and it has no killing power. It is likely that this component represents the protein "coat" of the virus, condensed about fragments of host DNA. The possibility that these particles might have transducing activity has not been tested.

The DNA of ϕX174 is atypical in that it reacts with formaldehyde, even when this reagent is applied to intact virus. As the usual double-stranded DNA molecules do not undergo this reaction, unless severely denatured, the DNA of ϕX174 must differ in structure from the hydrogen-bonded double helix proposed by Watson & Crick.

Further evidence that the DNA of ϕX174 may be unusual may be deduced from the measurements of DNA content reported here and the measurement by Tessman

(Tessman, Tessman & Stent, 1957; Tessman, personal communication) of inactivation of ϕX174 by decay of incorporated phosphorus-32. Measurements by Hershey, Kamen, Kennedy & Gest (1951) and by Stent & Fuerst (1955) have shown that in bacterial viruses the decay of a single incorporated phosphorus-32 atom can inactivate the virus, and that the chance that the decay of any given phosphorus atom will inactivate is a constant, α. For several bacterial viruses, at 4°C, α has been shown to be approximately 0·10.

Tessman's data for inactivation of ϕX174 by the decay of incorporated phosphorus-32 indicate that $\alpha = 1{\cdot}6 \times 10^6/$(DNA content of ϕX174). Since the DNA content is now known to be approximately $1{\cdot}6 \times 10^6$, α is, within the limits of the accuracy of the measurements, 1·0. A phosphorus disintegration within ϕX174 has ten times the efficacy observed with other bacterial viruses. It seems reasonable to attribute this unusual efficiency of inactivation to an unusual DNA structure.

It is a pleasure to indicate the technical assistance, at various times, of Mrs. Jean Sonder, Mrs. Alice White, Mrs. Agnes Gara, Mrs. Betsy Laties, Miss Faith Poole, and Miss Sharon Palmer. Antigenic studies of the 70*S* particles were made by Mr. George Guthrie. Drs. M. Meselson and J. Vinograd gave valuable assistance in the initial use of the density gradient sedimentation equilibrium method.

REFERENCES

Adams, M. H. (1950). *Methods in Medical Research*, Vol. 2. Chicago: The Year Book Publishers.
Bonét-Maury, P. & Bulgakov, N. (1944). *C.R. Soc. Biol., Paris*, **138**, 499.
Bulgakov, N. & Bonét-Maury, P. (1944). *C.R. Soc. Biol., Paris*, **138**, 497.
Dische, Z. (1930). *Mikrochemie*, **8**, 4.
Doty, P. & Steiner, R. F. (1950). *J. Chem. Phys.* **28**, 1211.
Fraenkel-Conrat, H. (1954). *Biochim. biophys. Acta*, **15**, 307.
Fraser, D. & Jerrel, E. A. (1953). *J. Biol. Chem.* **205**, 291.
Frilley, M., Bulgakov, N. & Bonét-Maury, P. (1944). *C.R. Soc. Biol., Paris*, **138**, 726.
Hershey, A. D., Dixon, J. & Chase, M. (1953). *J. Gen. Physiol.* **36**, 777.
Hershey, A. D., Kamen, M. D., Kennedy, J. W. & Gest, H. (1951). *J. Gen. Physiol.* **34**, 305.
Hoard, D. E. (1957). Ph.D. Thesis, University of California, Berkeley.
Lea, D. E. & Salaman, M. H. (1946). *Proc. Roy. Soc.* B, **133**, 434.
Lépine, P., Bonét-Maury, P., Bulgakov, N. & Giuntine, J. (1944). *C.R. Soc. Biol., Paris*, **138**, 728.
Markham, R. & Smith, J. D. (1954). *The Proteins*, Vol. II, Pt. A, ed. by H. Neurath & K. Bailey. New York: Academic Press.
Meselson, M., Stahl, F. W. & Vinograd, J. (1957). *Proc. Nat. Acad. Sci., Wash.* **43**, 581.
Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.
Schwerdt, C. E. (1957). *N.Y. Acad. Sci. Spec. Publ.* v, 157.
Sertic, V. (1937*a*). *C.R. Soc. Biol., Paris*, **124**, 14.
Sertic, V. (1937*b*). *C.R. Soc. Biol., Paris*, **124**, 98.
Sertic, V. & Bulgakov, N. (1935). *C.R. Soc. Biol., Paris*, **119**, 1270.
Staehelin, M. (1958). *Biochim. biophys. Acta*, **29**, 410.
Stent, G. S. & Fuerst, C. R. (1955). *J. Gen. Physiol.* **38**, 441.
Tessman, I. (1958). Personal communications.
Tessman, I., Tessman, E. S. & Stent, G. S. (1957). *Virology*, **4**, 209.
von Euler, H. & Hahn, L. (1946). *Svensk Kem. Tidskr.* **58**, 251.
Watson, J. D. & Crick, F. H. C. (1953). *Nature*, **171**, 737.
Webb, J. & Levy, H. B. (1955). *J. Biol. Chem.* **213**, 107.
Zahler, S. A. (1958). *J. Bacteriol.* **75**, 310.

J. Mol. Biol. (1959) **1**, 43-53.

A Single-Stranded Deoxyribonucleic Acid from Bacteriophage ϕX174†

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The deoxyribonucleic acid (DNA) of bacteriophage ϕX174 can be extracted by phenolic denaturation of the virus protein. The DNA thus obtained has a molecular weight of $1{\cdot}7 \times 10^6$, indicating that there is one molecule per virus particle.

This ϕX DNA does not have a complementary nucleotide composition. The ultraviolet absorption of this DNA is strongly dependent upon temperature in the range 20° to 60°C, and upon NaCl concentration in the range 10^{-3} to 10^0 M. This DNA reacts with formaldehyde at 37°C and is precipitated by plumbous ions. This evidence is interpreted to mean that the purine and pyrimidine rings are not involved in a tightly hydrogen-bonded complementary structure.

Light scattering studies indicate that this DNA is highly flexible and that its configuration is strongly dependent upon the ionic strength of the solution. Upon treatment with pancreatic deoxyribonuclease, the weight-average molecular weight decreases in accordance with the function expected for a single-stranded molecule.

It is concluded that the DNA of bacteriophage ϕX174 is single-stranded.

In a previous paper (Sinsheimer, 1959) describing the properties of bacteriophage ϕX174, evidence was presented that the deoxyribonucleic acid (DNA) of this virus appeared to be unusual, in that the amino groups of the purine and pyrimidine rings were accessible to reaction with formaldehyde, and in that the atomic efficiency of inactivation of the virus by disintegration of ^{32}P atoms, incorporated into the viral DNA, was 1·0 (Tessman, 1959). In the present paper chemical and physical studies of the DNA extracted from this virus are presented. The evidence obtained indicates that each ϕX174 particle contains one molecule of a single-stranded DNA.

Materials and Methods

Bacteriophage ϕX174 was prepared and purified as previously described.

Preparation of ϕX DNA

To a cold suspension of ϕX174 containing 10 to 15 mg/ml. in borate buffer ‡ is added an equal volume of cold phenol § (Gierer & Schramm, 1956) previously equilibrated with borate buffer by shaking. The resultant emulsion is shaken for 5 min in the cold room and then spun at 1800 *g* for 5 min to separate the phases. The aqueous phase is removed, added to an equal volume of cold phenol, and the process repeated two more times. The final aqueous layer which contains the DNA is removed and allowed to stand.

† This research has been supported by grants from the U.S. Public Health Service, the American Cancer Society, and the California Division of the American Cancer Society.

‡ Borate buffer is a solution of sodium tetraborate, saturated at 4°C.

§ Mallinckrodt liquified phenol, AR " intended for chromatographic purposes," is used.

To improve the yield of DNA a volume of borate buffer, equal to one-half the initial volume of ϕX suspension, is added to the phenol phase from the first extraction, shaken and spun as above. The aqueous layer is removed and then used to extract in turn the DNA from the phenol layers of the second and third extractions. The final aqueous layer is pooled with the DNA solution obtained in the first series of extractions, and the combined solutions are shaken with an equal volume of cold ether. After shaking the emulsion is allowed to stand in a separatory funnel for 1 hr to allow the phases to separate. The clear aqueous layer is drawn off. The turbid ether layer is then extracted with a few ml. of borate, which, after separation of the phases, is added to the DNA solution. Two more ether extractions are employed to remove most of the residual phenol.

At this stage the DNA content of the extract accounts for 65 to 70 % of the DNA in the initial suspension of virus.

The DNA is now dialyzed against two changes of 1·3 M-potassium phosphate buffer, pH 7·5 (Kirby, 1957). There is no loss of ultraviolet absorbing material upon dialysis. To complete the removal of residual denatured protein, the DNA is extracted from the salt solution by shaking with one-half volume of either 2-methoxyethanol or N,N-dimethyl-formamide (Porter, 1955). After vigorous shaking the two phases are separated by centrifugation at 1800 *g* for 8 min. The upper organic layer contains all the DNA. A thin film of denatured material appears at the interface of the two phases.

The DNA solution is then dialyzed against three changes of 0·2 M-sodium chloride plus 0·001 M-phosphate buffer, pH 7·5. 20 to 30 % of the ϕX DNA dialyses from the organic phase through the membrane into the first two changes.†

Methods of purine and pyrimidine analysis

Degradation to purines and pyrimidines was carried out with either 12 N-$HClO_4$ at 100°C (Marshak & Vogel, 1951) or with glass distilled 6 N-HCl at 100°C (Hershey, Dixon & Chase, 1953). With either method, the purines and pyrimidines recovered accounted for over 90 % of the phosphorus of the DNA preparation. Phosphorus analyses were made by a modification of the method of Allen (1940). Purines and pyrimidines were separated by descending chromatography in the isopropanol-water-HCl solvent of Wyatt (1951) using S. and S. 597 paper. In two instances this separation was checked, with good agreement, by a paper electrophoretic separation of the same digest, using 20 volts/cm at pH 3·2, 0·1 M-tris formate buffer, and Whatman no. 3 paper (Nutter & Sinsheimer, 1959). In all cases the purines and pyrimidines were eluted from the ultraviolet absorbing spots in 0·1 N-HCl and the quantities determined by measurement of the ultraviolet absorption, after subtraction of the absorption of appropriate blanks. The extinction coefficients employed were: adenine, A_M = 13,100 at 260 mμ (Beaven, Holiday & Johnson, 1955); guanine, A_M = 11,400 at 248 mμ (ibid.); thymine, A_M = 7,890 at 264·5 mμ (Shugar & Fox, 1952); and cytosine, A_M = 10,400 at 275 mμ (ibid.).

Degradation to nucleotides was accomplished by successive use of pancreatic deoxyribonuclease (DNase) (Worthington Biochemical Company) and venom phosphodiesterase (Koerner & Sinsheimer, 1957). Ion exchange fractionation of the nucleotides with 98 % recovery of the ultraviolet absorption was carried out as previously described (Sinsheimer & Koerner, 1951). Extinction coefficients for the nucleotides have been previously presented (Sinsheimer, 1954).

All spectra were obtained on a Beckman DK2 spectrophotometer.

Light scattering

All light scattering measurements were made in a Brice-Phoenix light scattering photometer, model 1000 D, made by Phoenix Precision Instruments, Inc. ϕX DNA solutions were cleaned of dust by filtration through type AA Millipore filters.

† This DNA can be recovered by evaporation of the pooled dialysate and solution in 0·2 M-NaCl plus 6 M-urea plus 0·001 M-versene, pH 7·0, followed by dialysis versus 0·2 M-NaCl. This DNA which has passed through the membrane has the same physical properties and composition as the DNA which remains within. It was at first thought that the escape of the ϕX DNA was a consequence of attack upon the membrane by methoxyethanol but the same result was observed with N,N-dimethyformamide and subsequent studies have indicated that the membranes are not permanently changed. Apparently ϕX DNA, in a largely organic medium, assumes a configuration in which it can slowly pass through the cellophane membrane.

Results

Composition of ϕX DNA

The results of several analyses of the purine and pyrimidine composition of two preparations of ϕX DNA are presented in Table 1. The nucleotide analysis achieved by enzymatic degradation is considered to be the most reliable. It is clear that the molar equalities of adenine and thymine and of guanine and cytosine observed in nearly all other DNA (Chargaff, 1955) are not observed. This DNA thus cannot have the complementary structure formulated by Watson & Crick (1953*a*). The only regularity that can be observed is that thymine/adenine = guanine/cytosine, but the significance of this is not immediately evident.

It appears that the methods of acid hydrolysis which have proven reasonably satisfactory for conventional forms of DNA are not entirely satisfactory for this DNA. A tendency to loss of thymine in $HClO_4$ hydrolysis (Wyatt, 1952) is exaggerated, as in the loss of adenine (Hershey, Dixon & Chase, 1953) in HCl hydrolysis.

Hydrogen bonding in ϕX DNA

The extracted DNA reacts with formaldehyde (Fig. 1) as does the native virus. This reaction is given by RNA (Fraenkel-Conrat, 1954; Staehelin, 1958) and by heat denatured DNA, but not by native DNA (Hoard, 1957). It is an indication that the amino groups of the purine and pyrimidine rings are accessible and are not involved in a tightly hydrogen bonded structure.

TABLE 1

Purine and pyrimidine composition of ϕX DNA

Preparation	Mode of degradation	Molar ratios			
		Adenine	Thymine	Guanine	Cytosine
A	6 N-HCl Hydrolysis	1·00	1·31	1·06	0·82
B	6 N-HCl Hydrolysis	1·00	1·31	1·06	0·82
B	12 N-$HClO_4$ Hydrolysis	1·00	1·06	0·99	0·76
B	Enzymatic digestion to nucleotides	1·00	1·33	0·98	0·75

Upon thermal denaturation of native DNA there is a disruption of the hydrogen bonded structure with a concomitant increase of ultraviolet absorption. With native DNA this transition takes place within a narrow temperature zone in the region of 80° to 90°C (Thomas, 1954; Lawley, 1956; Shack, 1958). Over the temperature range of 20° to 70°C there is very little influence of temperature upon the absorption of native DNA.

In contrast, the ultraviolet absorption of ϕX DNA is a marked function of temperature over a very wide range (Fig. 2). A temperature dependence of ultraviolet absorption of this nature is also observed with thermally denatured DNA (Shack, 1958), although the variation observed does not appear to be as great over the temperature region 20° to 50°C (Fig. 2). This variation of absorption with temperature is presumably the result, in part, of rupture of random intramolecular hydrogen bonds and, in part, a consequence of thermal expansion of the molecule.

Similarly, the ultraviolet absorption of ϕX DNA is strongly dependent upon ionic strength at values of ionic strength in which the absorption of native DNA is unaffected (Thomas, 1954; Lawley, 1956; Shack, 1958) (Fig. 3). Again the increase of absorption at lower ionic strength is presumably the result of decreased intramolecular hydrogen bonding and molecular expansion (*vide infra*) brought about by an increase of net charge upon the DNA.

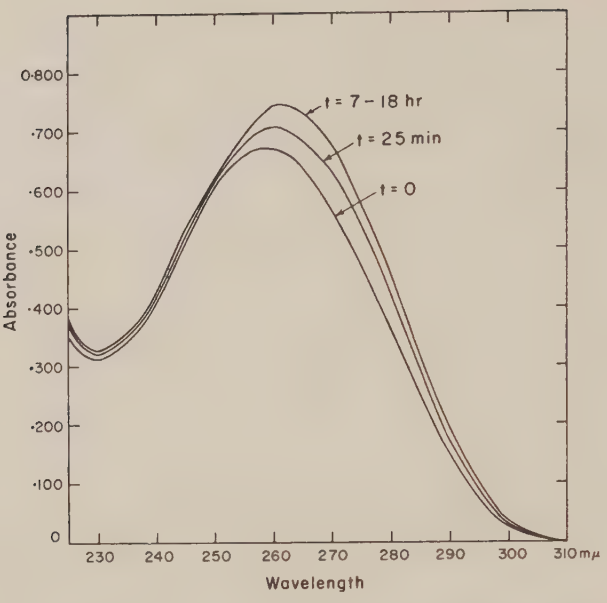


FIG. 1. Effect of formaldehyde (1·8 %) upon the ultraviolet absorption of ϕX DNA in 0·2 M-NaCl at 37°C. Concentration of DNA = 25·5 μg/ml.

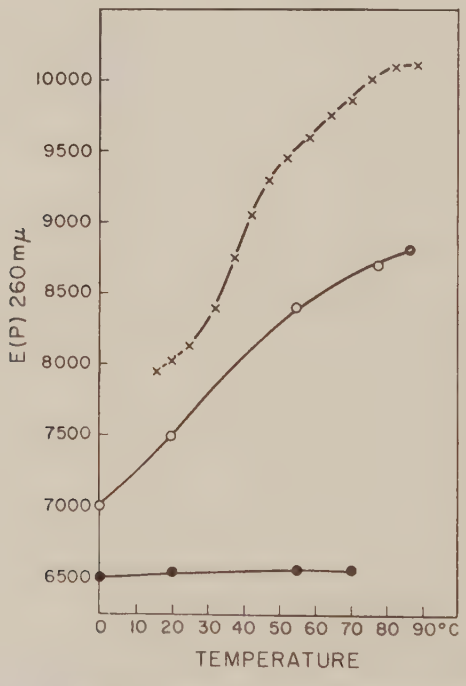


FIG. 2. Variation of the ultraviolet absorption of ϕX DNA at 260 mμ with temperature. $E(P)$ = absorbance of a solution containing one mole of phosphorus per liter.

x — x DNA from ϕX in 0·2 M-NaCl + 10^{-3} M-phosphate buffer, pH 7·5.

. — . Native thymus DNA in 0·1 M-NaCl (from Shack, 1958).

0 — 0 Heat denatured thymus DNA in 0·1 M-NaCl (from Shack, 1958).

It may be noted that $E(P)$, the absorbance per mole of phosphorus (Chargaff & Zamenhof, 1948), is 8700 for ϕX DNA in 0·2 M-NaCl at 37°C. This is considerably higher than has been observed with native or denatured DNA under comparable conditions. In accord with this observation, there is only an 11 % increase in the ultraviolet absorption of ϕX DNA upon degradation with pancreatic DNase in 0·2 M-sodium chloride at 37°C, as compared to the 30 to 35 % increase observed with native thymus DNA (Kunitz, 1950).

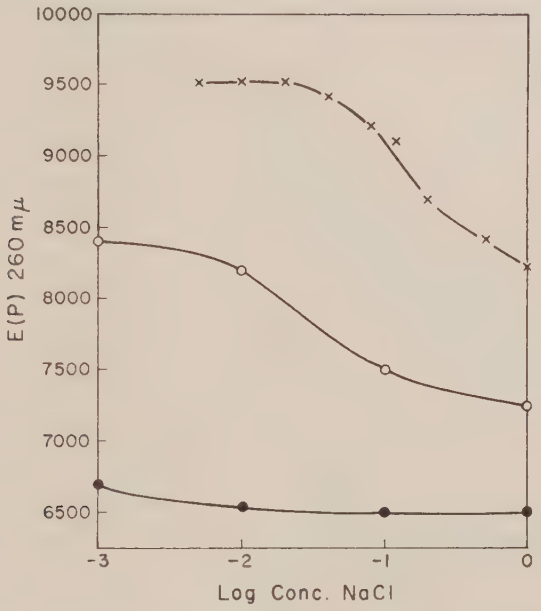


FIG. 3. Variation of the ultraviolet absorption at 260 mμ of ϕX DNA with salt concentration.

x — x DNA from ϕX, at 37°C.

. — . Native thymus DNA, at 22°C (from Shack, 1958).

0 — 0 Heat denatured thymus DNA, at 22°C (from Shack, 1958).

Stevens & Duggan (1957) showed that thermally denatured thymus DNA is precipitated by Pb^{++} ion under conditions such that native DNA remained soluble. Similarly, ϕX DNA is completely precipitated from a solution in 0·1 M sodium chloride containing 165 μg per ml. by addition of Pb^{++} ions to 0·017 M.

Light scattering observations

The particle weight of ϕX DNA has been measured by light scattering † (Figs. 4 and 5). Several preparations have been used under various ionic conditions and the weights obtained have always been within the range of $1{\cdot}6$ to $1{\cdot}8 \times 10^6$. This is in good agreement with the weight of DNA per ϕX particle calculated from the particle weight, $6{\cdot}2 \times 10^6$, and the DNA content, 25·5 %.‡ Hence there is one molecule of DNA per ϕX particle.

† In this calculation, the refractive increment of the DNA has been assumed to be 0·201 cm^3/g at 4358 Å (Northrop, Nutter & Sinsheimer, 1953), although this value may not be quite correct for a DNA of this type.

‡ The DNA content of ϕX was calculated from colorimetric analysis by the diphenylamine and *p*-nitrophenylhydrazine methods. In ordinary DNA these methods are believed to measure purine deoxyribosides (Dische, 1955). If this is still true with ϕX DNA, the total DNA content of ϕX will be slightly greater than 25·5 %, because the purine deoxyribosides comprise less than 50 % of the total DNA.

In 0·02 M-NaCl at 37°C the ϕX DNA has a radius of gyration of 1140 Å. Upon addition of salt to 0·2 M, this decreases to 440 Å (Fig. 6), while in 0·02 M-NaCl plus 4×10^{-3} M-Mg^{++} the radius of gyration is 325 Å.† ϕX DNA is thus a highly flexible

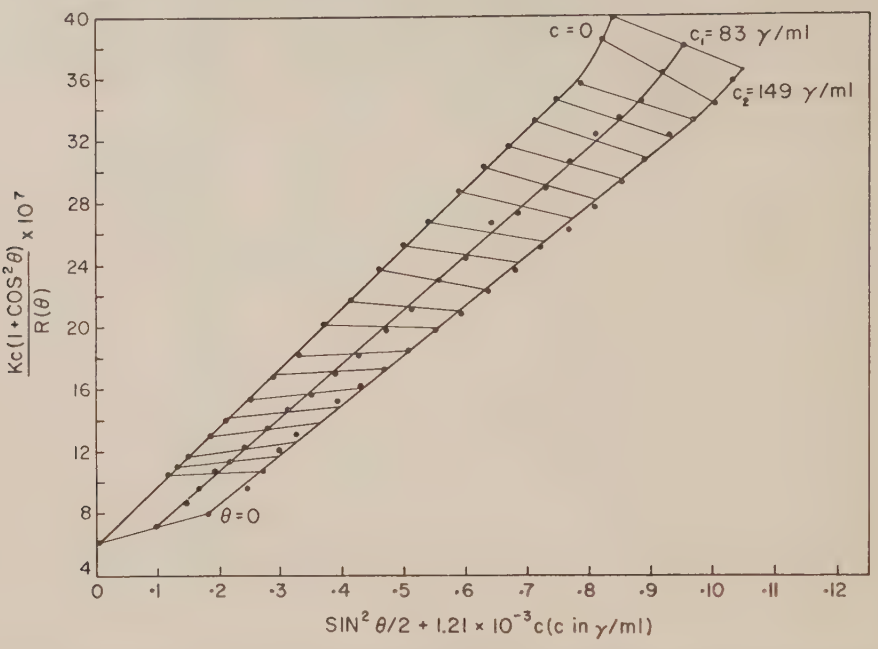


FIG. 4. Light scattering of ϕX DNA in 0·02 M-NaCl + 10^{-3} M-phosphate buffer, pH 7·5 at 37°C.

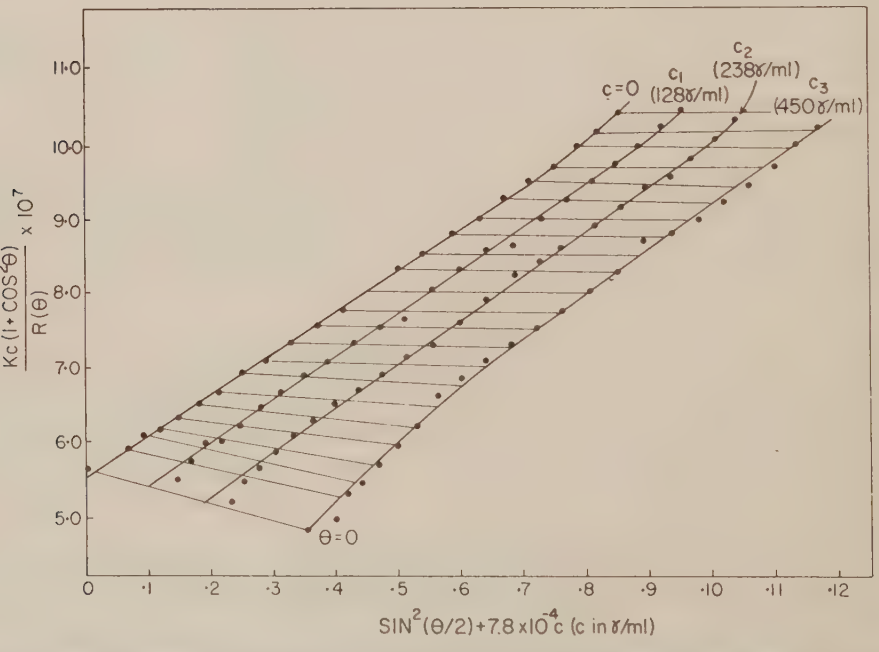


FIG. 5. Light scattering of ϕX DNA in 0·2 M-NaCl + 10^{-3} M-phosphate buffer, pH 7·5 at 37°C.

† At the same time, the ultraviolet absorption decreases by 14 % upon addition of Mg^{++}, and the sedimentation velocity increases (*vide infra*).

molecule. With native DNA, almost no change of radius of gyration is observed with change of ionic strength (Ehrlich & Doty, 1958). With alkali denatured DNA, a considerable variation is observed, nearly equal to that of ϕX DNA.

In 0·2 M-NaCl, the scattering envelope agrees well with that of a random coil (Doty & Steiner, 1950). For a random coil the radius of gyration should vary as $M^{0.5}$ (Tompa, 1956). For ϕX DNA in 0·2 M-sodium chloride, the ratio of $\sqrt{M}/\rho_g$ (M is the molecular weight in molecular weight units, ρ_g is the radius of gyration in Å) is 3·0. This may be

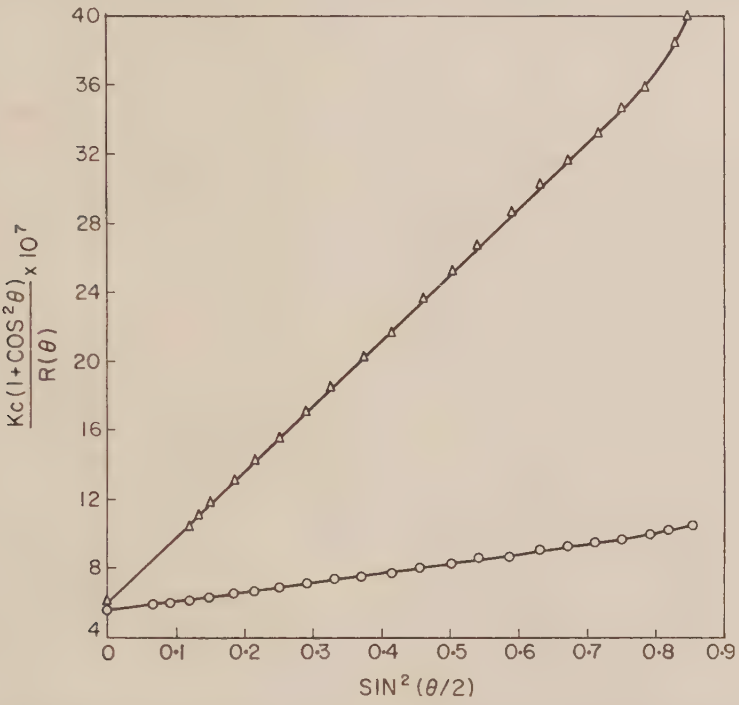


FIG. 6. Comparison of the scattering envelopes (extrapolated to zero concentration) of ϕX DNA in 0·02 M and 0·2 M-NaCl.

△ —— △ in 0·02 M-NaCl.
0 —— 0 in 0·2 M-NaCl.

compared with the ratio of 1·1 observed for native and sonicated thymus DNA (Reichmann, Rice, Thomas & Doty, 1954; Doty, McGill & Rice, 1958) and with a ratio of 2·5 to 2·6 observed with heat, acid, or alkali denatured thymus DNA in 0·2 M-sodium chloride (Rice & Doty, 1957; Thomas & Doty, 1956; Ehrlich & Doty, 1958). ϕX DNA is evidently more flexible than either native DNA or denatured DNA of thymus.

Kinetics of degradation by pancreatic deoxyribonuclease

As shown by Charlesby (1954) (also Tobolsky, 1957) the decline of the weight average molecular weight of a monodisperse single chain polymer upon random degration of the inter-monomeric links will be described by

$$\frac{M(p)}{M(O)} = \frac{2}{\gamma^2}[e^{-\gamma} + \gamma - 1]$$

where $M(p)$ is the weight average molecular weight when the probability of rupture of any link is p, $M(O)$ is the initial molecular weight and γ is proportional to p.

For a double chain structure, with cross linkage between the chains, the same equation applies except that γ is proportional to p^2, as shown by Thomas (1956).

Thomas and also Schumaker, Richards & Schachman (1956) have shown that the rate of splitting of bonds by pancreatic DNase is initially uniform with time ($p = kt$). Therefore, measurements were made of the decline with time (and thus with p) of the

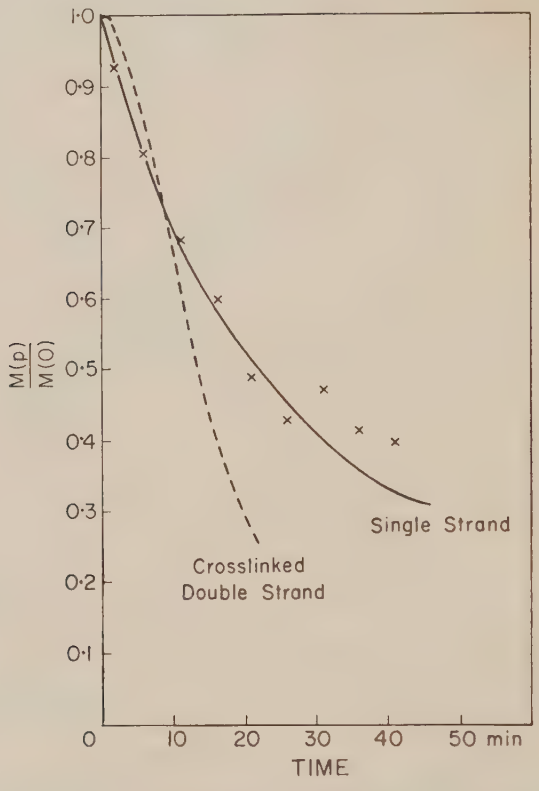


FIG. 7. Decline of weight-average molecular weight, as measured by light scattering, of ϕX DNA acted upon by pancreatic deoxyribonuclease. At $t = 0$, DNase in a final concentration of $3{\cdot}7 \times 10^{-7}$ μg/ml. was added to a solution containing 60 μg/ml. of ϕX DNA in 0·2 M-NaCl + 0·001 M-phosphate buffer, pH 7·5 + 0·02 M-magnesium acetate. The temperature was maintained at 34°C.

—— Function expected for a single-stranded molecule.

- - - - Function expected for a cross-linked double-stranded molecule.

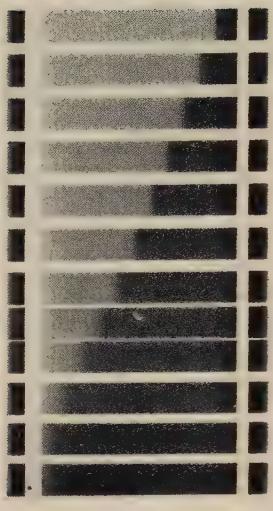
xxxx Experimental observations.

The theoretical functions were fitted to intersect a smooth curve representing the experimental data at one point, at $\frac{M(p)}{M(O)} = 0{\cdot}74$.

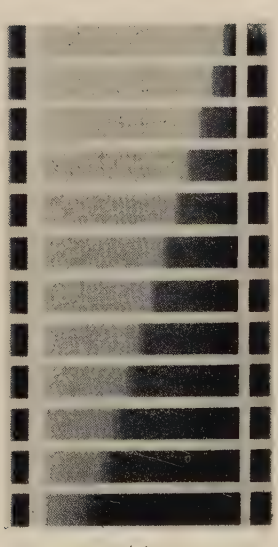
weight average molecular weight of ϕX DNA under attack by DNase. The form of the curve thus obtained (Fig. 7) agrees well with that expected for a single-stranded molecule and is clearly distinct from that expected for a two-stranded molecule with linked strands, such as native DNA.†

† On some occasions, with considerably lower concentrations of DNase, a slight curvature (concave downwards) has been observed in the initial stages of the digestion. This curvature, which was evident as an increase in slope to a value greater than the always finite initial slope, was not reproducible and has never been observed at the enzyme concentration employed in Fig. 7.

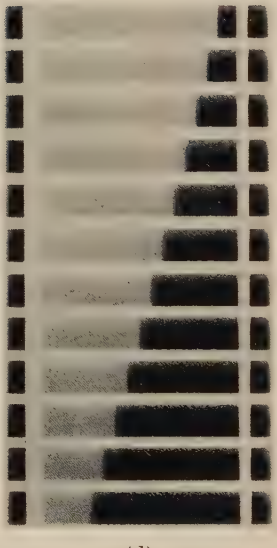
(a)



(b)



(c)



(d)

PLATE I (a). ϕX DNA and *E. coli* DNA banded in CsCl density gradient, after 20 hr at 44,770 rev/min. Density increasing to the right.

PLATE I (b). Ultracentrifuge sedimentation pattern of ϕX DNA (17 μg/ml.) in 0·2 M-NaCl at 20° C. Pictures taken every 4 min at 56,100 rev/min.

PLATE I (c). Ultracentrifuge sedimentation pattern of ϕX DNA (23 μg/ml.) in 0·04 M-NaCl at 20° C. Pictures taken every 4 min at 56,100 rev/min.

PLATE I (d). Ultracentrifuge sedimentation pattern at 20° C of ϕX DNA (32 μg/ml.) after 18 hr at 37° C in 1·8 % formaldehyde in 0·2 M-NaCl. Pictures taken every 4 min at 56,100 rev/min.

[*To face page* 51.

Ultracentrifuge studies

When banded by the cesium chloride density gradient equilibrium method of Meselson, Stahl & Vinograd (1957), ϕX DNA appears at a density of 1·72. This is distinctly higher than the density of native DNA (1·70) (Plate I (a)) and is very closely the density at which heat denatured salmon sperm DNA is observed to band (Meselson & Stahl, 1958).

In velocity centrifugation in 0·2 M-NaCl, ϕX DNA sediments as a single, rather sharp boundary, of $S = 23{\cdot}8$ (Plate I (b)). At lower ionic strength, the sedimentation rate decreases, and below 0·08 M-NaCl the boundary splits in two indicating the presence of two centrifugal components (Plate I (c)).

A similar splitting of the boundary can be observed in 0·2 M-sodium chloride after treatment with formaldehyde (Plate I (d)). The sedimentation constants of the two components are 15·8 and 13·9 (measured in the presence of formaldehyde). The

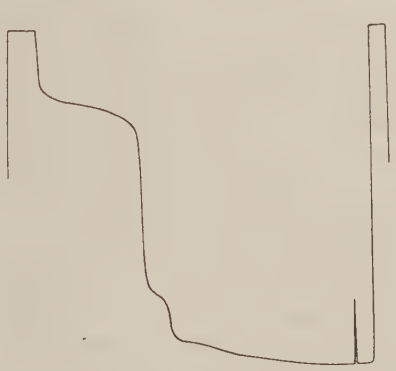


FIG. 8. Densitometer tracing of frame 11 of Plate I (d) indicating the relative proportions of the two centrifugal components.

relative proportions of the two components observed either in 0·04 M-sodium chloride or after formaldehyde treatment are the same for a given ϕX DNA preparation, although variations are observed in different preparations. The boundaries obtained after formaldehyde treatment are very sharp. A densitometer tracing is shown in Fig. 8.

The splitting of the boundary observed in dilute salt is completely reversible; upon addition of salt or Mg^{++} a single boundary is again observed.

Discussion

The chemical reactivity of ϕX DNA, its ultraviolet absorption properties, its density, and its flexibility as demonstrated by light scattering, all serve to distinguish the DNA of ϕX174 from conventional DNA. In several ways, such as the dependence of ultraviolet absorption upon temperature and ionic strength, and the dependence of the radius of gyration upon ionic strength, the properties of ϕX DNA appear to be an exaggeration of the properties of a denatured DNA, as contrasted to those of native DNA. The nucleotide composition rules out the possibility of a complementary structure, native or denatured. The simplest explanation of the properties observed is that the DNA of ϕX174 is a single-stranded structure. Such an explanation is also at least consistent with the high efficiency of inactivation of the ϕX virus by the decay of incorporated ^{32}P.

In order to test the possibility that some of the properties of ϕX DNA were artifacts produced by the method of extraction and purification, samples of native thymus and of native *E. coli* DNA were taken through the entire extraction and purification procedures. It was shown that these procedures have no effect upon the density of these DNA samples in the density gradient equilibrium method, or upon the increase in ultraviolet absorption observed when they were degraded with deoxyribonuclease. In addition the reactivity of ϕX DNA with formaldehyde before extraction from the virus may be cited.

The presence of two centrifugal components in the ϕX DNA preparation is not understood. They appear when the molecule is caused to take an extended form, either by destruction of intramolecular hydrogen bonds by formaldehyde or by lowering of the ionic strength. At one time it was thought that these might represent complementary strands of a DNA which had been separated biologically before incorporation into separate virus particles. However, it has been observed that the proportions of the two components varied from 62/38 to 79/21 in different preparations. Despite these variations, the preparations had identical nucleotide composition. This observation would appear to rule out the hypothesis of complementary chains.

The nucleotide composition of this DNA fulfils neither the adenine, thymine and cytosine, guanine equalities of ordinary DNA, nor the guanine plus uracil (thymine) equals adenine plus cytosine relation proposed for RNA by Elson & Chargaff (1954). In this respect, as in molecular size, this DNA is similar to the ribonucleic acid of the small plant viruses. It is, however, a DNA and differs notably from the RNA of TMV, for instance, with respect to stability and to several physical properties.

DNA fractions with compositions deviating from complementarity have been reported on other occasions (Lucy & Butler, 1954; Bendich, Pahl & Beiser, 1956). It might be very worth while to examine such fractions for the possible presence of single-stranded DNA components, by comparison of their properties with any of the distinctive properties of ϕX DNA. The existence of a single-stranded DNA would seem to imply the existence of a distinct mode of DNA replication (Watson & Crick, 1953*b*; Meselson & Stahl, 1958). It would be surprising if this were limited to a special class of bacterial viruses.

It is a pleasure to acknowledge the capable technical assistance of Miss Sharon Palmer.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955). Ch. 14 in *The Nucleic Acids*, Vol. I, ed. by E. Chargaff & J. N. Davidson. New York: Academic Press.
Bendich, A., Pahl, H. B. & Beiser, S. M. (1956). *Cold Spr. Harb. Symp. Quant. Biol.* **21**, 31.
Chargaff, E. (1955). Ch. 10 in *The Nucleic Acids*, Vol. I, ed. by E. Chargaff & J. N. Davidson. New York: Academic Press.
Chargaff, E. & Zamenhof, S. (1948). *J. Biol. Chem.* **173**, 327.
Charlesby, A. (1954). *Proc. Roy. Soc.* A **224**, 120.
Dische, Z. (1955). Ch. 9 in *The Nucleic Acids*, Vol. I, ed. by E. Chargaff & J. N. Davidson. New York: Academic Press.
Doty, P., McGill, B. B. & Rice, S. A. (1958). *Proc. Nat. Acad. Sci., Wash.* **44**, 432.
Doty, P. & Steiner, R. F. (1950). *J. Chem. Phys.* **18**, 1211.
Ehrlich, P. & Doty, P. (1958). *J. Amer. Chem. Soc.* **80**, 4251.
Elson, D. & Chargaff, E. (1954). *Nature*, **173**, 1037.
Fraenkel-Conrat, H. (1954). *Biochim. biophys. Acta*, **15**, 307.
Gierer, A. & Schramm, G. (1956). *Z. Naturf.* **11***b*, 138.

Hershey, A. D., Dixon, J. & Chase, M. (1953). *J. Gen. Physiol.* **36**, 777.
Hoard, D. E. (1957). Ph.D. Thesis, University of California, Berkeley.
Kirby, K. S. (1957). *Biochem. J.* 495.
Koerner, J. F. & Sinsheimer, R. L. (1957). *J. Biol. Chem.* 1049.
Kunitz, M. (1950). *J. Gen. Physiol.* **33**, 349.
Lawley, P. D. (1956). *Biochim. biophys. Acta*, **21**, 481.
Lucy, J. A. & Butler, J. V. (1954). *Nature*, **174**, 32.
Marshak, A. & Vogel, H. J. (1951). *J. Biol. Chem.* **189**, 597.
Meselson, M. & Stahl, F. W. (1958). *Proc. Nat. Acad. Sci. Wash.* **44**, 671.
Meselson, M., Stahl, F. W. & Vinograd, J. (1957). *Proc. Nat. Acad. Sci., Wash.* **43**, 581.
Northrop, T. G., Nutter, R. L. & Sinsheimer, R. L. (1953). *J. Amer. Chem. Soc.* **75**, 5134.
Nutter, R. L. & Sinsheimer, R. L. (1959). *Virology*, in press.
Porter, R. R. (1955). pp. 98-112 in *Methods in Enzymology*, Vol. I, ed. by S. P. Colowick & N. O. Kaplan. New York: Academic Press.
Reichmann, M. E., Rice, S. A., Thomas, C. A. & Doty, P. (1954). *J. Amer. Chem. Soc.* **76**, 3047.
Rice, S. A. & Doty, P. (1957). *J. Amer. Chem. Soc.* **79**, 3937.
Schumaker, V. N., Richards, E. G. & Schachman, H. K. (1956). *J. Amer. Chem. Soc.* **78**, 4230.
Shack, J. (1958). *J. Biol. Chem.* **233**, 677.
Shugar, D. & Fox, J. J. (1952). *Biochim. biophys. Acta*, **9**, 199.
Sinsheimer, R. L. (1954). *J. Biol. Chem.* **208**, 445.
Sinsheimer, R. L. (1959). *J. Mol. Biol.* **1**, 37.
Sinsheimer, R. L. & Koerner, J. F. (1951). *Science*, **114**, 42.
Staehelin, M. (1958). *Biochim. biophys. Acta*, **29**, 410.
Stevens, V. L. & Duggan, E. L. (1957). *J. Amer. Chem. Soc.* **79**, 5703.
Tessman, I. (1958). Personal communication.
Thomas, Jr., C. A. (1956). *J. Amer. Chem. Soc.* **78**, 1861.
Thomas, Jr., C. A. & Doty, P. (1956). *J. Amer. Chem. Soc.* **78**, 1854.
Thomas, R. (1954). *Biochim. biophys. Acta*, **14**, 231.
Tobolosky, A. (1957). *J. Polym. Sci.* **26**, 247.
Tompa, H. (1956). Polymer Solutions. London: Butterworths Scientific Publications.
Watson, J. D. & Crick, F. H. C. (1953*a*). *Nature*, **171**, 737.
Watson, J. D. & Crick, F. H. C. (1953*b*). *Nature*, **171**, 964.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.
Wyatt, G. R. (1952). *J. Gen Physiol.* **36**, 201.

J. Mol. Biol. (1959) **1**, 54-64

Twofold Symmetry of the β-lactoglobulin Molecule in Crystals

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The preparations of crystals of a cadmium derivative of β-lactoglobulin B and a *p*-chloromercuribenzoate derivative of β-lactoglobulin A are described.

A preliminary analysis of their X-ray diffraction patterns shows that in each case the molecule of molecular weight 35,000 is composed of two similar sub-units.

A description of the packing in the crystals is given in terms of a simple model of the molecule consisting of two spheres of 36 Å diameter in contact, with a twofold axis of symmetry between them.

Measurements of the molecular weight of β-lactoglobulin in solution at the isoelectric point by the methods of light-scattering (Halwer, Nutting & Brice, 1951), sedimentation-diffusion (Cecil & Ogston, 1949; Miller & Golder, 1952), and osmotic pressure (Bull & Currie, 1946) all lie within the range 35,000 to 36,000, as does the X-ray result from salt-free crystals (Senti & Warner, 1948). Since the discovery of the two genetically-controlled types of β-lactoglobulin (Aschaffenburg & Drewry, 1955), comparison of X-ray diffraction data from crystals of the individual allelomorphic proteins, now designated A and B (Aschaffenburg & Drewry, 1957), has shown that they are very similar in molecular weight, shape and general structure. They probably differ only in the substitution of different amino acids at a few sites within the molecule (Green, North & Aschaffenburg, 1956), and any general conclusions about the structure of the molecule, such as those drawn in this paper, should apply to both.

Under certain conditions the average molecular weight for the mixed A and B proteins isolated from bulk milk has been found to be about half the normal value. In surface films spread out on ammonium sulphate solutions Bull (1946) found mol. wt. $=17{,}000 \pm 490$, while Townend & Timasheff (1957) recently found approximately 17,500 in solutions below pH 3·5, both by light-scattering and sedimentation-diffusion methods. On the other hand there is evidence that the molecule of weight 35,000 can pair with others as the concentration of solutions at the isoelectric point is raised above about 0·5 g/100 ml. (Ogston & Tilley, 1955). This suggests, as Townend & Timasheff have remarked, that the fundamental unit of β-lactoglobulin has a molecular weight of only 17,500, though these fundamental units are normally associated in pairs, and show some tendency towards further aggregation.

Further support for this hypothesis can be found in the data for a few specific reactions of β-lactoglobulin with small molecules. There appear to be two N-terminal leucine (Fraenkel-Conrat, 1955) and two C-terminal *iso*leucine groups (Niu &

Fraenkel-Conrat, 1955) for mol. wt. = 35,000; two sulphydryl groups available to *p*-chloromercuribenzoate (PCMB) (Fraenkel-Conrat, Cook, and Morgan, 1952; Boyer, 1954); and, in the native molecule, approximately two sites for the combination of *n*-octylbenzene *p*-sulphonate (Hill & Briggs, 1956).

Even when there are strong suggestions from chemical and physico-chemical studies that a protein molecule is built up from identical sub-units, it may be difficult to find conclusive evidence of this from studies of the crystals of the protein. Each sub-unit may be expected to be a completely asymmetrical object, not superimposable on its mirror image, so the only type of symmetry element which can relate one sub-unit to another is the rotation axis, possibly coupled with translation to form a screw axis. Two sub-units may be linked in such a way as to have a twofold axis of symmetry between them, itself a rather unlikely situation, but then the pairs may pack into a crystal in such a way that the twofold symmetry does not reveal itself as crystallographic symmetry, i.e. it does not apply to the crystal as a whole. If the molecules pack with their twofold axes parallel or antiparallel, though, the symmetry may be revealed as a crystallographic dyad axis.

β-lactoglobulin provides examples of the various possible situations. The chemical evidence for sub-units is good, but the crystal forms so far characterized in the literature have all had at least one molecule of weight 35,000 in the asymmetric unit of pattern. In this paper two new crystal forms of derivatives of β-lactoglobulin are described: in one, the cadmium derivative of β-lactoglobulin B, molecular symmetry is revealed in a close approximation to a higher class of crystallographic symmetry; in the other, a compound of β-lactoglobulin A with *p*-chloromercuribenzoate, the asymmetric unit of pattern contains only a half-molecule of weight 17,500. Both were originally prepared in the hope of obtaining heavy-metal-containing crystals structurally isomorphous with the native protein.

Cadmium Derivative of β-Lactoglobulin B

Preparation and chemical analysis

Préaux, Hulsmans & Lontie (1954) were the first to show that a crystalline derivative of the β-lactoglobulins may be obtained by the addition of cadmium ions to a dialysed, casein-free filtrate prepared from bulk milk. From their subsequent work (Lontie & Préaux, 1955; Préaux & Lontie, 1956) it appears that crystals enriched in β-lactoglobulin B are formed by their procedure. Using solutions prepared from freeze-dried, salt-free crystals of β-lactoglobulin A or B, or mixtures of the two, we found that highly insoluble amorphous precipitates are formed when cadmium is added to A, and that a part of the proteins of mixed preparations is similarly precipitated under enrichment in A, whilst the supernatants yield crystals enriched in B on standing.

Addition of cadmium to β-lactoglobulin B does not result in any immediate visible changes, but after some hours copious formation of lozenge-shaped crystals occurs. For good crystal growth, a weight corresponding to 700 mg of moisture-free protein was dissolved in 10 ml. of 0·1 M-acetate buffer, pH 5·8, and the solution was made up to 100 ml. with water and filtered. To 5 ml. portions of the filtrate (= 1 μmole protein), cadmium was added in the form of 0·002 M solutions of the sulphate or acetate (0·5 ml. = 1 μmole). This leads to a reduction in pH. The yield of crystals

increases rapidly until, with additions of 1·5 ml. of the cadmium solution, about 70 to 80% of the protein crystallizes out. For some reason the rate of crystal formation is greatly accelerated when the solutions are covered with toluene. This effect is not due to simple exclusion of air, as layering with liquid paraffin, for instance, has no such effect on the growth rate.

For analytical purposes, crystals were developed in 15 ml. centrifuge tubes. The solutions were seeded and crystallization was allowed to proceed for at least 24 hr. The crystals were then spun down, and the clear supernatant removed for measurement of the residual protein content by ultra-violet absorption at 278 mμ and calculation of the crystallized protein by difference. The cadmium content of the crystals was determined by direct complexometric titration following the procedure given by Schwarzenbach (1957): a few drops of his ammonia buffer of pH 10 were used to dissolve the crystals. The solution was then washed into a conical flask

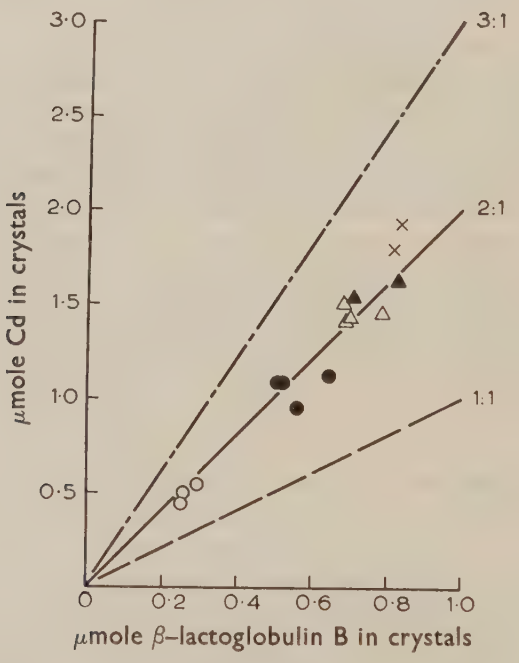

Fig. 1. Binding of cadmium by β-lactoglobulin B(1 μmole). (○ = 1, ● = 2, △ = 3, ▲ = 4, × = 5 μmole Cd added.)

with water, warmed, and titrated with 0·001 M-ethylenediamminetetra-acetate in the presence of Eriochrome Black T. Sharp endpoints were obtained in spite of the high dilution, and no interference from the protein was noted. No correction was made for any cadmium present in the supernatant adhering to the centrifuged crystals; washing of the crystals with water led to erratic titration results. Analytical results obtained in experiments in which 1 to 5 μmole cadmium were added to 1 μmole of β-lactoglobulin B are shown in Fig. 1. They leave little doubt that the number of cadmium atoms bound per molecule of the protein is two. This ratio tends to be exceeded when 5 or more μmole of the metal are added to 1 μmole of protein, probably because of further unspecific binding. The site of binding is not known; possibly the metal combines with sulphydryl groups of which the protein seems likely to contain two (Fraenkel-Conrat *et al.*, 1952; Boyer, 1954).

Although single crystals of satisfactory size for X-ray crystallography were sometimes obtained after growth in centrifuge tubes, they could be developed with greater ease by allowing crystallization to proceed in long glass tubes of diameter of about 0·5 cm which were held undisturbed in a near-horizontal position. An increased number of single crystals was formed under these conditions, though clustering still occurred.

Crystallography

Crystals of cadmium β-lactoglobulin B from preparations made by adding one or four cadmium atoms per molecule of protein were examined, with similar results. The crystals appear as monoclinic plates bounded by (100), (010) and (001) faces, looking very much like the crystals of native β-lactoglobulin B illustrated in Aschaffenburg & Drewry (1955). In preparations which are exposed to the air for a day or two these crystals dissolve slowly and are replaced by tetragonal needle-shaped crystals having the cell dimensions given in Table 1(*c*).

X-ray diffraction spectra have been recorded for the monoclinic form covering the [100], [001], [010], and [10$\bar{1}$] zones out to a Bragg spacing of 3·5 Å, using the Buerger precession camera. The cell dimensions are given in Table 1(*a*), followed in Table 1(*b*) by the cell dimensions of the monoclinic form of native β-lactoglobulin B, to which the cadmium derivative is evidently closely related. The similarity of the cell volumes is so close that one may be certain there are two molecules of weight 35,000 to each cell without repeating the measurements of crystal density and water content which were made for the native protein (Green *et al.*, 1956).

Although, strictly, both crystals have monoclinic symmetry, space-group $P2_1$ there is a striking pseudo-symmetry in the intensities of the spectra from the cadmium derivative which, though hinted at, is not so evident with the related form of the native protein. Plate I illustrates this with a reproduction of the (*h*0*l*) spectra. In this (*h*0*l*) plane, as an example, the only requirement of the true monoclinic symmetry is that the intensities of opposite spectra should be equal, $|F(h0l)|^2 = |F(\bar{h}0\bar{l})|^2$, but the rule $|F(h0l)|^2 = |F(l0h)|^2$ is rather closely obeyed in addition: for the reflections of Plate I

$$\frac{\sum \left| |F(h0l)|^2 - |F(l0h)|^2 \right|}{\sum \left| |F(h0l)|^2 + |F(l0h)|^2 \right|} = 0{\cdot}11$$

If this symmetry were perfect, the crystal could be assigned unambiguously to a more symmetrical crystallographic class—orthorhombic, space group $B22_12$. As it is, the approximation is so close that it is worth examining the relationship of the pseudo-space group to the true monoclinic one. The symmetry operations of space group $B22_12$ are illustrated in Fig. 2 in terms of a schematic arrangement of units which are bounded by a spherical surface but are completely asymmetrical in detailed internal structure. This asymmetry is symbolized by inscribing tetrahedra with numbered vertices in the spheres. The arrangement is not close-packed, but neighbouring units are in contact. In the figure the outlines of the spheres and the numbers at the vertices are shown as seen in projection: a negative sign over any number indicates that the vertex is below the centre of its sphere. For the relationships of neighbouring spheres it is simplest to take them first in pairs one above the other: these have been drawn somewhat displaced in Fig. 2. Each pair has a twofold axis of symmetry

relating the vertices of the two tetrahedra. The pairs are then arranged at the intersections of an oblique net to form a layer, all pairs having the same orientation. Below this layer is a precisely similar one rotated about the layer normal through 180°: indicated with broken-line circles in Fig. 2. The layer below this is identical with the first, so completing the repeat in the direction normal to the diagram. With this arrangement extended indefinitely through space, five sets of crystallographic symmetry elements arise: four sets lying parallel to the plane of the diagram are shown with double-headed arrows for twofold axes and single-headed arrows for twofold screw axes; while a further set of twofold screw axes, indicated by ◊, runs normal to the figure. The unit cell of this arrangement would normally be chosen as a rectangular block with one face centred, containing eight asymmetrical units; but a primitive cell containing only four asymmetrical units, and with one pair of axes not enclosing a right angle, would also be a valid choice. The outlines of these cells on the centred face are shown in the figure.

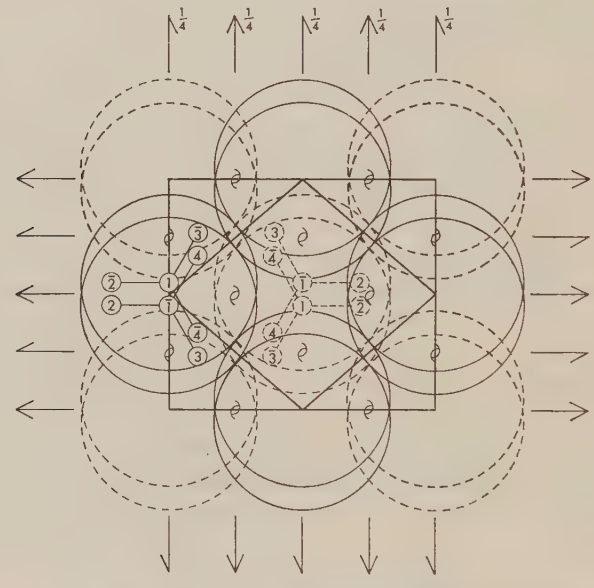

Fig. 2. Arrangement of asymmetrical sub-units in the pseudo-orthorhombic cell of cadmium β-lactoglobulin B. For explanation see text.

The arrangement of Fig. 2 has perfect orthorhombic ($B22_12$) symmetry; a small distortion of the arrangement reduces the symmetry to monoclinic ($P2_1$). Fig. 3(*a*) is a simplified version of Fig. 2: the pairs of asymmetric units have been reduced to symbolic figures-of-eight with their polar twofold axes attached. In Fig. 3(*b*) the pairs have been rotated slightly about the normal to the figure: this preserves the screw axis symmetry in that direction, but the symmetry in the other directions, formerly represented by the twofold axes parallel to the plane of the figure, has been destroyed. This arrangement is then monoclinic, with the oblique unit cell still containing four of the spheres, but with two now making up each asymmetric unit of pattern. The X-ray spectra from a crystal of this type can show pseudo-orthorhombic symmetry only if the distortion from the situation of Fig. 3(*a*) is small.

Fig. 2 is intended to serve not merely as an illustration of symmetry relationships but also as an idealized model of the molecular arrangement in crystals of monoclinic

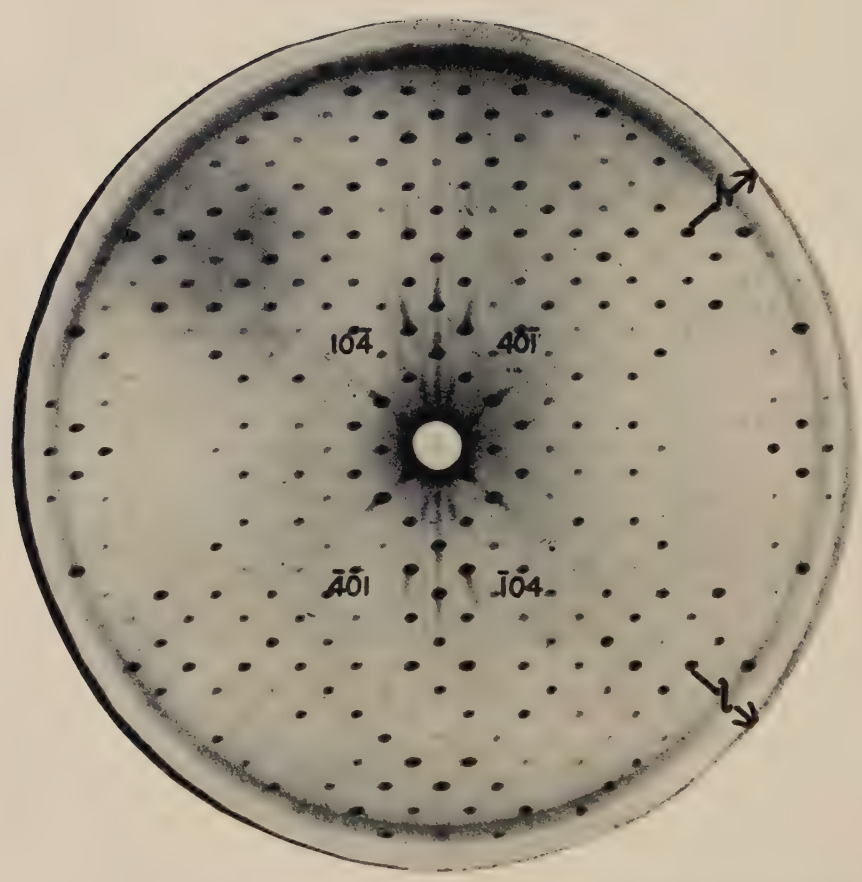

PLATE I. 13° precession photograph of the $h0l$ spectra of cadmium β-lactoglobulin B.

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β-lactoglobulin B and its cadmium derivative. It remains to identify the asymmetrical units in molecular terms, and to assign dimensions to the model and check them against the measured crystal cell parameters. The monoclinic cell of either crystal contains two molecules of weight 35,000, so the pseudo-orthorhombic, rectangular, face-centred cell of the cadmium derivative must contain four of these molecules. We have seen, though, that the truly orthorhombic centred cell is required to contain eight asymmetric units. The solution of this dilemma is to have the 35,000 molecular weight molecule made up of two sub-units which are joined so as to have a twofold axis of symmetry between them. The imperfection of the crystal symmetry may be

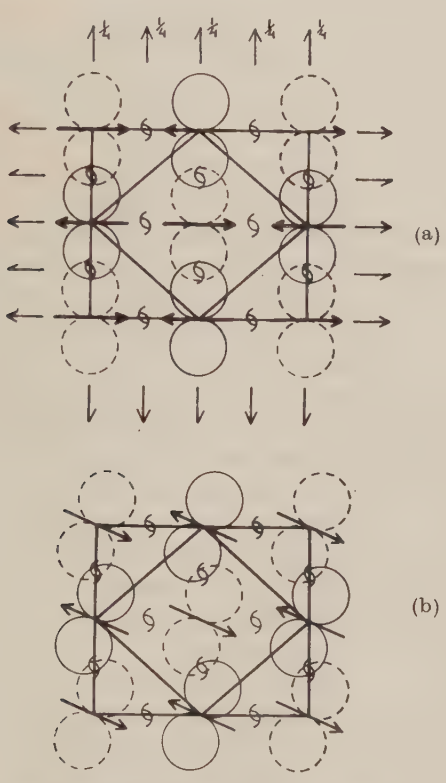

FIG. 3. Reduction of orthorhombic symmetry (a) to monoclinic (b), by a rotation of the molecules in Cd β-lactoglobulin B.

due to any of three things: possibly the sub-units are identical, but the molecular twofold axis is not precisely aligned with the crystallographic dyad (as suggested above); possibly they are identical but not joined with perfect twofold symmetry; or, possibly, they are slightly different. Any difference must be quite small: an estimate from the measured asymmetry of the ($h0l$) reflections suggests an equivalent molecular weight difference of only 250 units. The method used to arrive at this figure is given in the following paragraph.

The real structure R is to be regarded as the sum of a symmetrical structure S and a perturbation structure P containing comparatively little scattering matter (P may include negative scattering matter). The observed scattering factors will be sums of those from S and P

$$F_R = F_S + F_P$$

If F_P is sufficiently small, the phases of F_R and F_S may be taken to be the same at all reciprocal lattice points, so

$$|F_P| = \Big||F_R| - |F_S|\Big|$$

Now the scattering power of the perturbation structure will be proportional to $\sum |F_P|^2$, while that of the real structure is proportional to $\sum |F_R|^2$. The approximate proportionality of scattering power and molecular weight enables one to estimate the molecular weight of the perturbation structure as $\frac{\sum |F_P|^2}{\sum |F_R|^2} \times 35{,}000$ per molecule of β-lactoglobulin. In other words, if the sub-units differ only in molecular weight, then they differ by this amount. Using the $(h0l)$ spectra of Plate I, one finds $\frac{\sum |F_P|^2}{\sum |F_R|^2} = 0{\cdot}007$, so the molecular weight difference is approximately 250 units.

We now know that, as far as symmetry is concerned, we can identify the sub-units of the molecule with the asymmetric units of Fig. 2; is it also reasonable to represent them as spheres? If so, the a- and c-axial lengths give the sphere diameter as 36·4 Å. Taking the two units of a pair to be precisely one above the other, and the contact between layers to be as shown, the length of the b-axis, normal to the figure, can be calculated to be 127·6 Å, which is the measured value.

A further check can be applied to the $P2_1$ form of the native protein, which has similar cell dimensions, but does not show the pseudo-symmetry so clearly, possibly because the molecular twofold axes are turned further away from the pseudo-dyad direction. For this crystal, measurements of the density and water content are available. From the data provided in Green *et al.*, (1956) one may calculate that, of the cell volume of 164,000 Å^3, 96,000 Å^3 is occupied by dry protein and 68,000 Å^3 by water. Each of the four sub-units in the cell occupies 24,000 Å^3 which is the volume of a sphere 35·8 Å in diameter. Since it is likely that the molecular packing volume in the wet crystal includes some bound water, this is in satisfactory agreement with the size estimated from the cell dimensions.

Although the model is satisfactory in both these ways, it is, of course, highly idealized, and unrealistic in several respects. In the crystal it does not provide a sufficient number of contacts between one molecule and its neighbours to fix the angle between the a- and c-axes, and the single point contact within the pair does not suggest the stability of the 35,000 molecular weight unit. However, a small distortion of the spherical surface, increasing the diameter by 2·3 Å in the right direction, is enough to bring each pair of spheres into contact with twelve other pairs, and the narrow "waist" of the molecule can be filled in to provide a reasonable area of contact without changing the volume greatly. The real molecule may be expected to be a distortion and elaboration of the two-spheres model in other ways too, for it is likely to have a very irregular surface, but an axial ratio of 2:1 and a twofold axis of symmetry at right angles to the length appear obligatory.

It should be noted that another delineation of the molecule can be made without changing any of these conclusions: in Fig. 2 the molecular twofold axis has been drawn parallel to the larger diagonal of the oblique face of the primitive cell, but the molecule may be chosen from one asymmetric unit in the plane drawn with solid lines, and an adjacent unit in the plane drawn with broken lines; the molecular twofold axis is then parallel to the shorter diagonal of the oblique face.

PCMB Derivative of β-Lactoglobulin A

Preparation

As PCMB is known to interact with the mixed β-lactoglobulins isolated from bulk milk (Fraenkel-Conrat *et al.*, 1952; Boyer, 1954), attempts were made to prepare the products of its reaction with the separate β-lactoglobulins in the crystalline state. With β-lactoglobulin B, all attempts failed: when treated in the way described below for A, the protein was evidently modified so as to form products of much increased solubility. Dialysis at or near the iso-electric point of the native protein no longer led to the separation of an oil or of solid matter.

With β-lactoglobulin A, the solubility of the reaction products was also higher than that of the starting material, and rose with increasing addition of the mercurial. However, using concentrated protein solutions, oils and, eventually, crystals were obtained. For this purpose, solutions containing 35 mg protein per ml. were made in 1 M-acetate buffer of pH 5·2, and volumes of a 2·5 mM solution of PCMB corresponding to one to four equivalents of the mercurial were added. The PCMB was dissolved in 0·5 N-NaOH, and its pH reduced with HCl to faint turbidity which was then cleared by addition of alkali (final pH 8·0 to 8·5). On standing, the solution tended to throw a precipitate, and it was prepared afresh for each experiment. The mixtures of protein and PCMB remained clear when left to interact for 24 hr at room temperature. They were then dialysed against water in narrow bags of 8/32 in. Visking tubing, and formed oils within 24 hr. The quantity of oil decreased from bags containing PCMB and protein in the ratio 1 : 1 to those containing the 4 : 1 mixture. Only the former produced crystals on further dialysis; at higher ratios the oils could not be induced to crystallize even after seed-crystals had become available. Oils from the 1 : 1 mixture were slow to change into crystals, several days being required for completion compared to a few hours with oils of the native protein.

Single crystals of adequate size for X-ray studies were difficult to prepare by the above procedure. Better results were obtained by use of the sodium salt of *p*-chloromercuribenzene-sulphonic acid (PCMBS) which, being more soluble at pH values below 7, is also easier to handle than PCMB. Furthermore, it permits the use of a weaker acetate buffer for the dissolution of the protein. With this mercurial, 35 mg of β-lactoglobulin A were dissolved in 1 ml. of 0·1 M-acetate buffer, and the requisite amounts of PCMBS added in the form of a 0·002 M solution. When dialysis was started after interaction for 24 hr, the solutions began to precipitate oil within the hour, and the process was completed after 3 to 4 hr. As with PCMB, only oils from 1 : 1 mixtures could be induced to change into the crystalline state. When seeded they produced rather small crystals, and much better specimens were obtained by adding seeds at an earlier stage when the solutions showed the first signs of precipitating oil. Even so, the crystals, which were found to be isomorphous with those prepared with the aid of PCMB, could not be grown to anything like the size attainable with the native protein.

Crystallography

Crystals of the PCMB derivative of β-lactoglobulin A, or the PCMBS derivative, appear as rather flat rectangular prisms bounded by (100), (010) and (001) faces, and show straight extinction under the polarizing microscope. In preparations which

are left exposed to the air, these crystals dissolve, being replaced after a day or two by elongated prismatic crystals terminated in a point by four dome faces. The cell dimensions of this second form are shown in Table 1(e), but it will not be discussed further here, as it gives no indication of molecular symmetry.

TABLE 1

Unit cell dimensions of crystal forms of β-lactoglobulin

	Name	Space group	Cell dimensions					Mols.†		Notes
			a (Å)	*b* (Å)	*c* (Å)	*β*	$V \times 10^{-5}$ (Å^3)	/cell	/as.u.	
(a)	Cd β-lg B	$P2_1$	36·4 ± 0·1	127·6 ± 0·4	36·4 ± 0·1	98° 12′ ± 10′	1·68	2	1	Pseudo-$B22_12$
(b)	β-lg B	$P2_1$	36·1 ± 0·1	127·5 ± 0·4	36·0 ± 0·1	106° 05′ ± 10′	1·64	2	1	
(c)	Cd β-lg B	$P4_22_1$	69·2 ± 0·3	—	138·8 ± 1·0	—	6·65	8	1	
(d)	PCMB β-lg A and PCMBS β-lg A	$P22_12_1$ or $P22_12$	35·4 ± 0·5	66·9 ± 0·8	71·7 ± 1·0	—	1·70	2	½	
(e)	PCMB β-lg A and PCMBS β-lg A	$P2_12_12_1$	62·8 ± 0·5	73·4 ± 0·3	148·6 ± 0·5	—	6·86	8	2	

† The figures on the left give the number of molecules per unit cell, those on the right the number per asymmetric unit, in each crystal form.

Because the original crystals, which are rather small, dissolve so quickly when they are mounted in a capillary, it has proved impossible to obtain more than a small sample of diffraction data from them. Only photographs with the crystal stationary or oscillating through 5° on the precession camera, have been completed. These are sufficient to establish the cell dimensions given in Table 1 (d), and to show that the crystals are orthorhombic, but too few reflections have been observed to be sure if the space group is $P22_12_1$ or $P22_12$. Whichever it is, a general argument similar to that used for the cadmium derivative of β-lactoglobulin B shows that the molecule of β-lactoglobulin A is also geometrically divisible into sub-units. Just as a face-centred orthorhombic pattern such as $B22_12$ requires eight asymmetric units per cell to satisfy the symmetry, so a primitive orthorhombic arrangement like $P22_12_1$ or $P22_12$ must have four asymmetric units in the cell. But the unit cell of PCMB β-lactoglobulin A, with a volume of 170,000 Å^3, can accommodate only two molecules of weight 35,000, so, again, the asymmetric units have to be identified with structural sub-units, two of them related by a twofold axis of symmetry making up the molecule.

The model of two 36Å diameter spheres can be fitted in among the symmetry elements of either space group. Fig. 4(a) and 4(b), which are projections down the short *a*-axis, show the model molecule and the *b*- and *c*- cell edges drawn to scale: in 4(a) the arrangement has $P22_12_1$ symmetry, in 4(b) it is $P22_12$.

If the space group is $P22_12_1$ the molecular twofold axis must be parallel to the crystallographic *a*-axis, which is only 35·4 Å long—just about the width of the suggested molecule. However, the *b*- and *c*- axes are considerably longer than one would predict from the closest possible packing of spheres. The relevant contacts are

all of the same type, each end of a molecule fitting into the space between the "waists" of two others. The situation would be improved by expanding the "waist" in the direction at right angles to the twofold axis; but in the direction of the axis it can remain thin, and probably is so because the asymmetrical polypeptide structure must not cross the line of twofold symmetry.

If the space group is $P22_12$, two orientations are possible for the molecular dyad either along the crystallographic a-axis as shown in Fig. 4(b), or along the c-axis, in which case the molecule is chosen differently from the same arrangement of sub-units. The structure is not so close-packed as with the $P22_12_1$ space group, so the

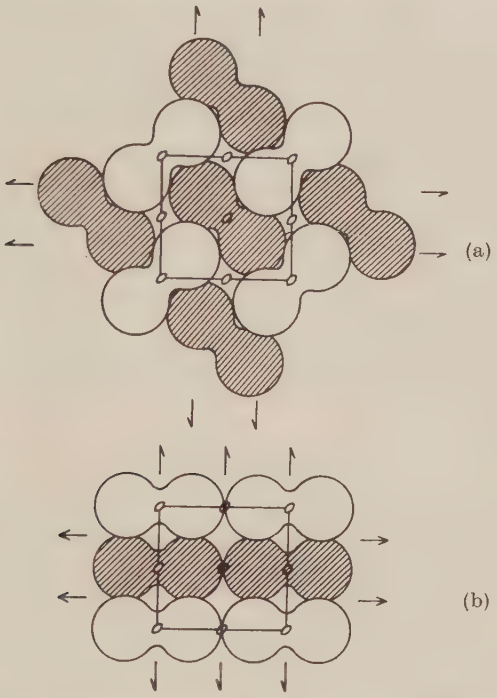

FIG. 4. Possible molecular arrangements in the unit cell of PCMB β-lactoglobulin A: (a) symmetry $P22_12_1$ (b) symmetry $P22_12$.

situation of contacts between neighbours is less satisfactory, but the cell dimensions predicted from the packing of perfectly spherical sub-units are in better agreement with those observed for the actual crystal.

The model developed for the cadmium derivative of β-lactoglobulin B is thus not in conflict with the crystallography of PCMB β-lactoglobulin A, though it may need to be expanded a little in some directions. The two crystal forms discussed in this paper are the simplest yet reported for β-lactoglobulin and appear to be the most promising for detailed crystallographic study.

REFERENCES

Aschaffenburg, R. & Drewry, J. (1955). *Nature*, **176**, 218.
Aschaffenburg, R. & Drewry, J. (1957). *Nature*, **180**, 376.
Boyer, P. D. (1954). *J. Amer. Chem. Soc.* **76**, 4331.
Bull, H. B. (1946). *J. Amer. Chem. Soc.* **68**, 745.
Bull, H. B. & Currie, B. T. (1946). *J. Amer. Chem. Soc.* **68**, 742.

Cecil, R. & Ogston, A. G. (1949). *Biochem. J.* **44**, 33.
Fraenkel-Conrat, H. (1955). *Symposium on Structure of Enzymes and Proteins*, p. 153. Gatlinburg, Tennessee.
Fraenkel-Conrat, J., Cook, B. B. & Morgan, A. F. (1952). *Arch. Biochem. Biophys.* **35**, 157.
Green, D. W., North, A. C. T. & Aschaffenburg, R. (1956). *Biochim. biophys. Acta*, **21**, 583.
Halwer, M., Nutting, G. C. & Brice, B. A. (1951). *J. Amer. Chem. Soc.* **73**, 2786.
Hill, R. M. & Briggs, D. R. (1956). *J. Amer. Chem. Soc.* **78**, 1590.
Lontie, R. & Préaux, G. (1955). 3e *Colloque de l'Hôpital St. Jean, Bruges*, p. 177.
Miller, G. L. & Golder, R. H. (1952). *Arch. Biochem. Biophys.* **36**, 249.
Niu, C.-I & Fraenkel-Conrat, H. (1955). *J. Amer. Chem. Soc.* **77**, 5882.
Ogston, A. G. & Tilley, J. M. A. (1955). *Biochem. J.* **59**, 644.
Préaux, G., Hulsmans, J. & Lontie, R. (1954). *Arch. int. Physiol.* **62**, 574.
Préaux, G. & Lontie, R. (1956). *Proc. XIVth Int. Dairy Congr.* **3**, 439.
Schwarzenbach, G. (translated by H. Irving). (1957). *Complexometric Titrations*, p. 83. London, Methuen and Co.
Senti, F. R. & Warner, R. C. (1948). *J. Amer. Chem. Soc.* **70**, 3318.
Townend, R. & Timasheff, S. N. (1957). *J. Amer. Chem. Soc.* **79**, 3613.

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Calcification of Collagen

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The paper deals with a study of the deposition of calcium phosphate in the form of apatite crystallites inside collagen fibres. By treating collagen fibres with a suspension of precipitated calcium phosphate, an excellent precipitation of hydroxyapatite inside the collagen was obtained. The X-ray diffraction pattern of the treated specimen showed a striking similarity to that of a section of normal bone. The lines were sharper in the pattern of the treated specimen indicating a large particle size in the precipitate. Addition of citric acid in small amounts was found to affect the particle size and the X-ray pattern was found to correspond more closely to the bone pattern. Increased addition of citric acid inhibited the deposition of apatite while a still larger concentration of citric acid resulted in the crystallization of calcium citrate itself. The presence of other salts like calcium carbonate and calcium chloride suppressed the deposition of apatite. In the case of calcium fluoride a mixed crystallization of fluoride and apatite took place if the calcium fluoride was present in equal concentration, while the presence of excess of calcium fluoride was found to completely suppress the deposition of both apatite and fluoride.

1. Introduction

It was observed in this laboratory that a number of soluble inorganic salts could be crystallized inside collagen fibres by keeping the fibres in solutions of these salts (Ramachandran & Ambady, 1955). The condition for the deposition seems to be that the repeat spacing along the collagen fibre axis must correspond to a multiple of one of the parameters of the crystallizing material. Some experiments carried out by the author (unpublished) showed that relatively insoluble substances also had a tendency to crystallize inside collagen under certain conditions. In view of this, an attempt was made to deposit calcium phosphate in the form of apatite inside collagen fibres by treatment with suitable solutions containing calcium and phosphate ions. Such a study would be of great help in the understanding of the mechanism of bone formation. After a number of failures it was finally possible to obtain a deposit whose X-ray pattern resembled that of natural bone. This paper gives an account of these studies. The work was completed about two years ago, but only a brief note was published then (Ramachandran & Santanam, 1956).

Although a large amount of work has been done on the composition of bone salt and the nature of the crystallizing material, one still cannot say that we have a clear picture in all its detail. Several review articles have been published (Huggins, 1937; Fankuchen, 1945; Dallemagne, 1950; Clark & Iball, 1957), and so only a brief review of the present state of knowledge is given in this section.

(a) *Electron-microscopic studies on bone*

The size and shape of bone crystals and collagen–crystal relationships in bone were demonstrated by Wolpers (1943) and Robinson (1952). Robinson & Watson

(1952) suggested that the apatite is present in the form of tabular crystals and the dimensions of these crystals were suggested as 350 Å × 300 Å × 50 Å. However Fernández-Morán & Engström (1957) have suggested that the apatite particles are needle shaped, 30–60 Å in diameter with an average length of about 200 Å, aligned parallel to the collagen fibres.

(b) *X-ray diffraction studies on bone*

Although there is general agreement regarding the similarity of the structure of bone crystals and the apatite minerals, various suggestions have been put forward concerning the composition of bone salts. At present there are three main schools of thought regarding the inorganic constituent of bone, claiming that it is (1) α-tricalcium phosphate together with free carbonate (Dallemagne, 1952); (2) a carbonate–apatite (McConnell, 1952), and (3) hydroxyapatite adsorbed on the surface of the crystals (Hendricks, 1952). On the basis of X-ray diffraction data, Clark & Iball (1957) have suggested a unit cell for bone with $a = 9{\cdot}37$ Å and $c = 6{\cdot}88$ Å.

(c) *Biochemical aspects of calcification*

There is general agreement in the biochemical literature that the composition of bone salts undergoes wide variations, although the same apatite structure is found to occur in all specimens. It has also been suggested that calcium secondary phosphate is a first step in the formation of bone salts, as also tertiary calcium phosphate (Shear & Kramer, 1928 ; Logan & Taylor, 1947). The chief objection to the theory has been that secondary calcium phosphate has never been revealed by X-ray methods (Huggins, 1937). Sobel & Burger (1954*a*,*b*) and Sobel & Hanok (1952), from a study of the reversible inactivation of calcification *in vitro* by strontium and beryllium, have suggested the involvement of chondroitin sulphate in the calcifying mechanism.

2. Experimental Results

Attempts were made to precipitate the calcium phosphate on collagen fibres by keeping them in a mixture of equal parts of 0·1 M-calcium chloride solution and 0·1 M secondary sodium phosphate solution. The flocculent precipitate of calcium phosphate formed under these conditions did not enter the collagen lattice, as shown by the X-ray pattern. The precipitation was carried out in the presence of ammonia, as also small amounts of sodium chloride or sodium carbonate, but in all these cases no positive results were obtained. A solution of primary sodium phosphate (0·1 M-NaH_2PO_4) was then tried with calcium chloride and in this case the treated fibres gave the pattern given in Plate I (d). The salt that was crystallized inside the fibres has been identified as calcium secondary orthophosphate, $CaHPO_4 \cdot 2H_2O$. The spacings and intensities of the pattern agreed completely with the data reported for calcium secondary orthophosphate in A.S.T.M. index card No. 2-0085. A buffer mixture of the primary and secondary phosphate with the pH adjusted to a value of 7·6 was also tried, but with no success. In the presence of small amounts of sodium fluoride added to the calcium chloride, calcium fluoride was seen to crystallize inside the fibre. The pattern of such a specimen is given in Plate I (f). The spacings and intensities agreed with those reported for calcium fluoride in A.S.T.M. index card No. 4-0864. Instead of calcium chloride, Ringer solution was also tried without

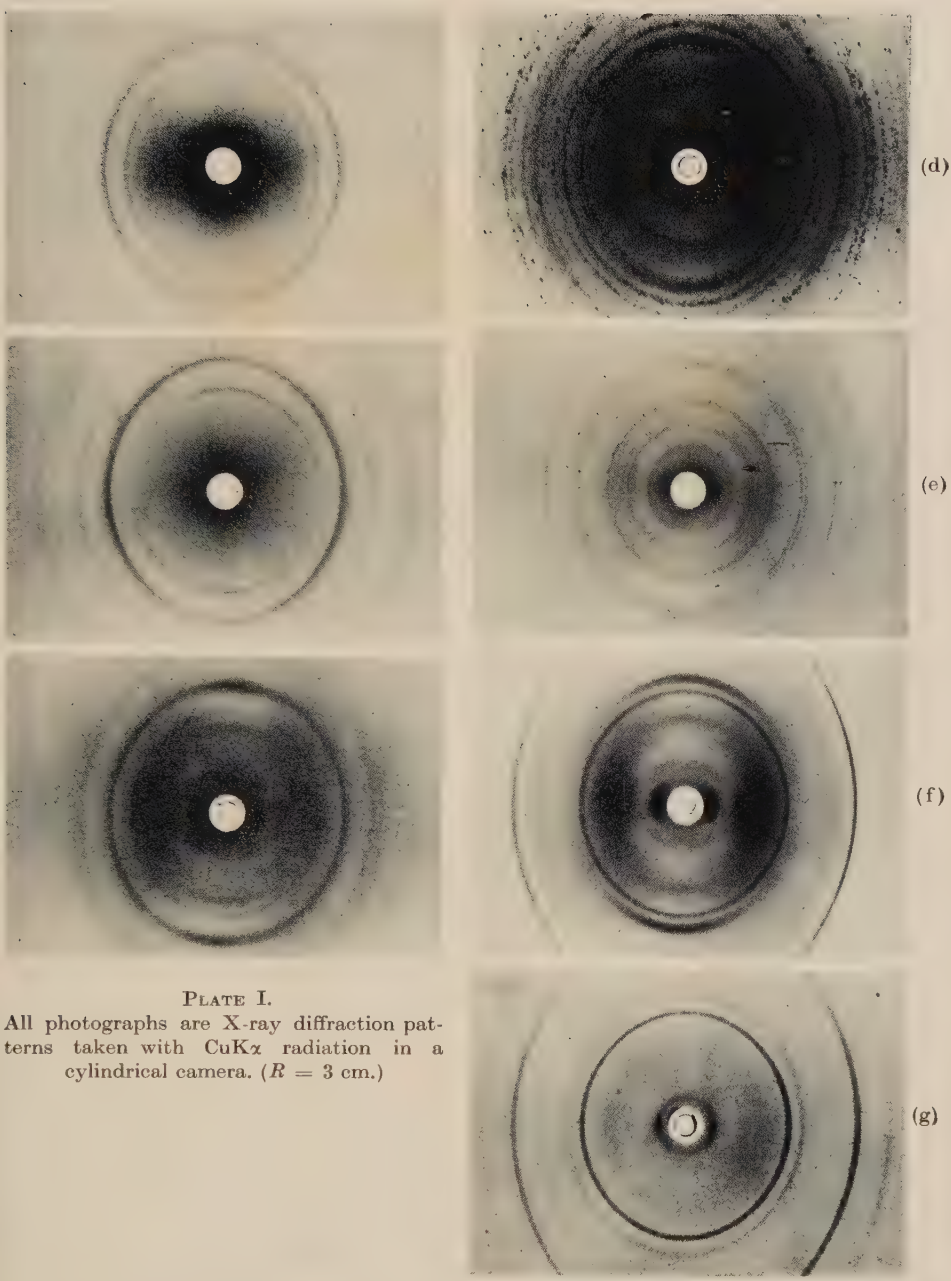

PLATE I.

All photographs are X-ray diffraction patterns taken with CuKα radiation in a cylindrical camera. (R = 3 cm.)

(a) Collagen treated with a suspension of precipitated calcium phosphate (B.D.H.). (b) Section of bone. (c) Collagen treated with a suspension of precipitated calcium phosphate to which citric acid has been added. (d) Collagen deposited with calcium secondary orthophosphate. (e) Collagen deposited with calcium citrate. (f) Collagen deposited with calcium fluoride. (g) Collagen showing mixed deposition of apatite and fluorite.

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success. Addition of adenosine triphosphate to the solution was also found to be ineffective.†

Finally, a 10 % suspension of precipitated calcium phosphate (B.D.H.) was tried and the pattern of the treated specimen is given in Plate I (a). This pattern agreed almost completely with the pattern of the specimen of precipitated calcium phosphate used for the suspension (not reproduced). However, the interesting fact is that the pattern shows a remarkable similarity to the pattern of a section of bone (Plate I (b)). A comparison of the two patterns showed however minor differences in intensities, but not in spacings (Table 1). Also the lines are sharper in the pattern of the treated specimen than in the bone pattern, indicating that the particle size of hydroxyapatite is much larger in the precipitate than in natural bone. Hendricks (1952) has suggested that, in bone, carbonate and citrate ions occur essentially on the surface of hydroxyapatite crystallites and that their occurrence limits the particle size. Consequently experiments were tried by passing carbon dioxide through the suspension and adding citric acid to it. Passing carbon dioxide did not have any effect on the deposition except that the amount of the crystallizing substance was slightly reduced. Addition of citric acid in small amounts (of the order of 0·02 mg/ml.) was found to affect the particle size and the optimum effect was found by adding 0·02 mg/ml. of citric acid, with the pH adjusted to 7·6 by addition of 10 % ammonia. The pattern is given in Plate I (c). The spacings and intensities of these patterns are compared with those found for bone and those reported for hydroxyapatite in the A.S.T.M. index in Table 1.

TABLE 1

Comparison of X-ray patterns of bone, treated specimens and hydroxyapatite

Spacings from bone		Spacings from the fibre treated with precipitated calcium phosphate to which citric acid has been added		Spacings from the fibre treated with the suspension of precipitated calcium phosphate		Spacings for hydroxyapatite from A.S.T.M. index card No. 1–1008	
d	Intensity	*d*	Intensity	*d*	Intensity	*d*	Intensity
3·46 Å	M.S	3·46 Å	M.S	3·46 Å	M.S	3·44 Å	40
3·07	M	3·07	M	3·11	M	3·11	8
—	—	—	—	2·92	W	—	—
2·80	S	2·80	S	2·80	S	2·79	100
2·67	W	2·67	W	2·67	W	2·62	8
2·30	M	2·30	M	2·30	M	2·27	16
2·14	V.W	2·14	V.W	2·14	V.W	2·13	4
2·05	V.W	2·05	V.W	2·05	V.W	2·06	4
1·97	M	1·97	M	1·97	M	1·94	20
1·83	M	1·83	M	1·83	M	1·84	20
1·71	W	1·71	W	1·71	W	1·71	16

Further addition of citric acid (2 mg/ml.) completely inhibited the deposition of apatite crystallites and the pattern was found to be similar to that of ordinary collagen, without any deposition of inorganic salt. Still further increase of citric acid (10 mg/ml.) caused the deposition of calcium citrate tetrahydrate (Plate I (e)). The spacings and intensities of the pattern agreed with those reported for calcium citrate tetrahydrate reported in A.S.T.M. index card No. 3–0038.

† It may be mentioned that some of these observations are in agreement with the findings of Glimcher, Hodge & Schmitt (1957) during their studies of the mineralization of collagen.

The influence of the presence of other salts on the deposition of apatite was also studied. Addition of calcium chloride and calcium carbonate was found to suppress the deposition of apatite. When calcium fluoride was added, a mixed crystallization of both the salts occurred (Plate I (g)). The pattern showed both the lines of fluorite and apatite. The presence of calcium fluoride in excess (10 : 1) completely suppressed the deposition of apatite and the pattern showed only the collagen diagram. No calcium fluoride was deposited, although when only calcium fluoride was used in the suspension this salt crystallized inside the fibre.

With a view to verifying the ideas of Sobel *et al.* that chondroitin sulphate is involved in the calcifying mechanism, deposition of apatite was carried out after prior treatment of the collagen fibres with a concentrated solution of calcium chloride to remove the chondromucoids. Still the deposition took place and an identical pattern to that in Plate I (c) was obtained.

3. Conclusions

The studies reported here indicate that it is possible to deposit calcium phosphate in the form of apatite on collagen fibres as such by treating them with a suspension of precipitated calcium phosphate. The particle size is affected by addition of citric acid. The addition of citric acid beyond an optimum limit inhibits the deposition of calcium phosphate. It is of interest to recall here that whenever citrate accumulates locally, calcium if available is found to accumulate in the form of slightly ionized calcium citrate itself (Hastings, 1955). The mixed crystallization of apatite and fluoride may be of interest in the understanding of fluorosis.

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REFERENCES

Clark, S. M. & Iball, J. (1957). *Progr. Biophys.* **7**, 226.
Dallemagne, M. J. (1950). *Annu. Rev. Physiol.* **12**, 101.
Dallemagne, M. J. (1952). *Trans. Macy Conf. Metabol. Interrel.* **4**, 154.
Fankuchen, I. (1945). *Annu. Rev. Biochem.* **14**, 207.
Fernández-Morán, H. & Engström, A. (1957). *Biochim. biophys. Acta*, **23**, 260.
Glimcher, M. J., Hodge, A. J. & Schmitt, F. O. (1957). *Proc. Nat. Acad. Sci., Wash.* **43**, 860.
Hastings, A. B. (1955). *Ann. N.Y. Acad. Sci.* **60**, 798.
Hendricks, S. B. (1952). *Trans. Macy Conf. Metabol. Interrel.* **4**, 185.
Huggins, C. (1937). *Physiol. Rev.* **17**, 119.
Logan, M. & Taylor, H. L. (1947). *J. Biol. Chem.* **79**, 147.
McConnell, D. (1952). *Trans. Macy Conf. Metabol. Interrel.* **4**, 169.
Ramachandran, G. N. & Ambady, G. K. (1955). *Experientia*, **11**, 343.
Ramachandran, G. N. & Santanam, M. S. (1956). *Experientia*, **12**, 340.
Robinson, R. A. (1952). *J. Bone Jt. Surg.* **34**A, 389.
Robinson, R. A. & Watson, M. L. (1952). *Anat. Rec.* **114**, 383.
Shear, M. J. & Kramer, B. (1928). *J. Biol. Chem.* **79**, 147.
Sobel, A. E. & Burger, M. (1954*a*). *Fed. Proc.* **13**, 300.
Sobel, A. E. & Burger, M. (1954*b*). *Proc. Soc. Exp. Biol., N.Y.* **87**, 7.
Sobel, A. E. & Hanok, A. (1952). *J. Biol. Chem.* **197**, 669.
Wolpers, C. (1943). *Klin. Wschr.* **22**, 624.

J. Mol. Biol. (1959) **1**, 69-80

The Structures of Ferritin and Apoferritin: Some Preliminary X-Ray Data

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Ferritin is a protein containing about 20 % by weight of iron in the form of a micelle of ferric hydroxide: this is surrounded by a shell of apoferritin. The iron can be removed and the apoferritin crystallised as colourless octahedra similar to the brown octahedral crystals formed by ferritin.

An X-ray study has been made of horse spleen apoferritin and of two crystalline forms of ferritin A and B from the same source. Apoferritin and ferritin B crystals are isotropic, while those of ferritin A are anisotropic. All three crystals have identical lattice repeats; wet crystals of apoferritin and ferritin B are face-centred cubic with $a = 184$ Å, while ferritin A is only pseudo-cubic, being truly face-centred on only one face. The lattice of ferritin A can therefore be described in terms of an orthorhombic unit cell with $a = 130$ Å, $b = 130$ Å, $c = 184$ Å. None of the space-groups are determined unambiguously from the X-ray data, but depend on the exact configurations of the molecules. The space group of apoferritin is probably $F'432$, the unit cell containing four molecules. This being the case each molecule should have point group symmetry 432, and hence be composed of $24n$ identical sub-units, where n is an integer. A possible molecular structure is suggested, that of 24 sub-units situated at the vertices of a snub-cube. In ferritin B the protein structure is probably identical with that of apoferritin, but point groups 432, $43m$ or $m3m$ are allowable for the iron hydroxide micelle. If, as suggested by electron microscopy, the micelle consists of four particles at the vertices of a tetrahedron, or eight particles at the vertices of a cube, then each of these particles is probably composed of three sub-units. The possibility that the observed symmetries of apoferritin and ferritin B are produced statistically is also discussed briefly. The X-ray data for ferritin A are consistent with the molecules containing a single two-fold axis, the space group of the orthorhombic unit cell being $P2_12_12$, $P2_122_1$ or $P22_12_1$. Thus either there are two species of ferritin in which the micelles are oriented differently with respect to the protein, or the lattice of ferritin A does not use the full symmetry of the molecule.

A molecular diameter of 111–112 Å is derived for apoferritin and ferritin A, the air-dried crystals having a face-centred cubic arrangement with a respectively 158 Å and 156 Å.

A molecular weight of 747,000 is obtained for ferritin A. If certain assumptions are made, a molecular weight close to that obtained from sedimentation-diffusion studies can be calculated for apoferritin.

Introduction

Ferritin is an unusual protein containing a very large proportion of iron (about 20 % of its dry weight). It was first isolated from horse spleen by Laufberger (1937) who showed that it could be crystallised readily from cadmium sulphate in the form of characteristic brown octahedral crystals.

The bulk of the iron is in the ferric state. It can be removed by the action of reducing agents such as sodium dithionite (Granick & Michaelis, 1943), cysteine, glutathione

and ascorbic acid (Biclig & Bayer, 1955*a*; Mazur, Baez & Shorr, 1955) and the iron-free protein, apoferritin, crystallises from cadmium sulphate as colourless octahedral crystals closely similar to those of ferritin. The ferric iron is in the form of colloidal ferric hydroxide micelles (Michaelis, 1947) which make up about 36 % by weight of the molecules. There is strong evidence in support of the view that the micelles are situated at the centres of the molecules (Farrant, 1954; Mazur & Shorr, 1950; Kuff & Dalton, 1957), each micelle being surrounded by a shell of protein. It may be concluded that free apoferritin is simply this protein shell, the central region now being empty or replaced by the liquid in which the protein is immersed.

Apoferritin moves in the ultracentrifuge with a sharp boundary, and sedimentation-diffusion studies have shown it to have a molecular weight of 465,000 (Rothen, 1944). An ellipsoidal shape was deduced for the molecule, axial ratio 1 : 3. The molecular weight of ferritin, however, cannot be estimated accurately by this method. Ferritin sediments with a widely spread boundary, varying in composition from colourless iron-free protein to coloured iron-containing complex with average weight 2·8 times that of the free apoferritin.

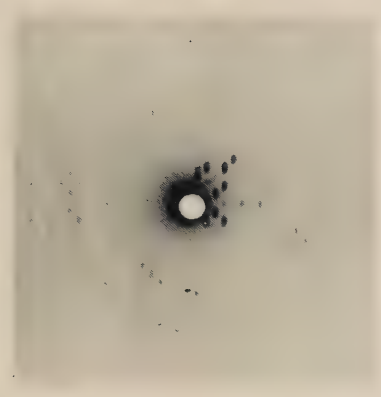
Early X-ray powder photographs obtained by Fankuchen (1943) indicated that ferritin and apoferritin were cubic and isomorphous, the cube edge in the wet crystals being 186 Å in length. The wet crystal densities were reported to be 1·45 and 1·27 respectively, and from these values, together with the microscopically measured linear shrinkage, anhydrous molecular weights of 460,000 for apoferritin and 545,000 for ferritin were derived, assuming 8 molecules in the cubic unit cell. It was later reported by Hodgkin (1949) and by the present author (Cowan, 1953) that the single crystals of ferritin, which they had examined, were orthorhombic, not cubic, wet unit cell dimensions being

$$a = 130 \text{ Å}, \qquad b = 130 \text{ Å}, \qquad c = 184 \text{ Å}.$$

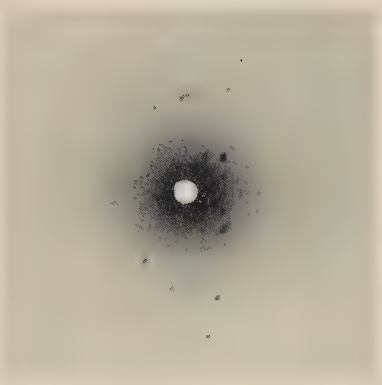
The space group $P2_12_12_1$ was derived for these crystals, but it was later concluded that this was not the true space group. A diameter about 50 Å was also deduced from the X-ray diffraction data for the heavily-scattering iron hydroxide micelles.

The whole molecule was shown by Farrant (1954) to measure about 100 Å across. The electron micrographs of horse spleen ferritin also showed that the molecule has a central core of highly electron-absorbing material about 55 Å across, which can be presumed to be the iron hydroxide micelle. The micelle is not uniform and consists of about four particles; the protein also apparently contains sub-units. Dense particles of the same size have also been observed in homogenates and in thin sections of cells from a wide range of organs (Kuff & Dalton, 1957; Richter, 1957, 1958*a*,*b*; Bessis & Breton-Gorius, 1957; Novikoff, Beaufay & de Duve, 1956; Palade & Siekevitz, 1956). Particularly interesting is the observation by Bessis & Breton-Gorius (1957) and by Richter (1958*a*,*b*) that haemosiderin granules consist of aggregates of dense particles, the particles in some granules being arranged on a lattice resembling that of a crystal of purified ferritin. These electron microscope studies have also confirmed Farrant's finding that the micelles are composed of about four smaller particles of diameter about 27 Å. There is some further evidence (Richter, 1957) that the actual building units are still smaller, about 8 Å across. Electron micrographs of carbon replicas of surfaces of ferritin crystals have been obtained by Labaw & Wyckoff (1957) and from these it has been deduced that the roughly spherical molecules, of diameter 109 Å, are arranged in cubic close-packing, the tetramolecular unit cube edge being 154 Å.

(a)



(b)



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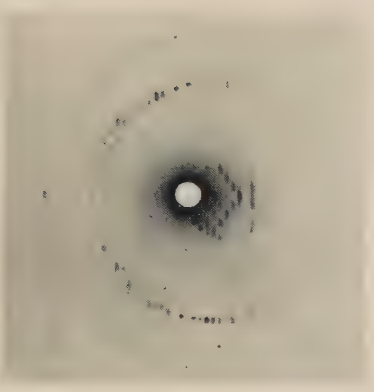

(d)

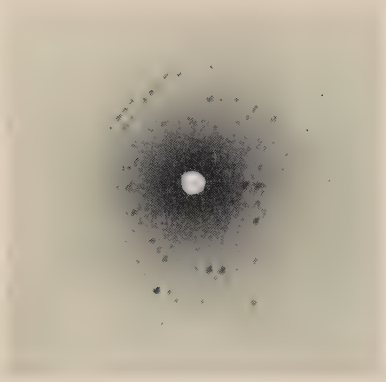

PLATE I. 2° oscillation X-ray diffraction photographs of ferritin A. Indices with the suffix *c* relate to the pseudo-cubic cell.

(a) X-ray beam parallel to $[001]_c$. $[110]_c$ direction vertical.
(b) X-ray beam parallel to $[010]_c$. $[101]_c$ direction vertical.
(c) X-ray beam parallel to $[1\bar{1}0]_c$. $[110]_c$ direction vertical.
(d) X-ray beam parallel to $[10\bar{1}]_c$. $[101]_c$ direction vertical.

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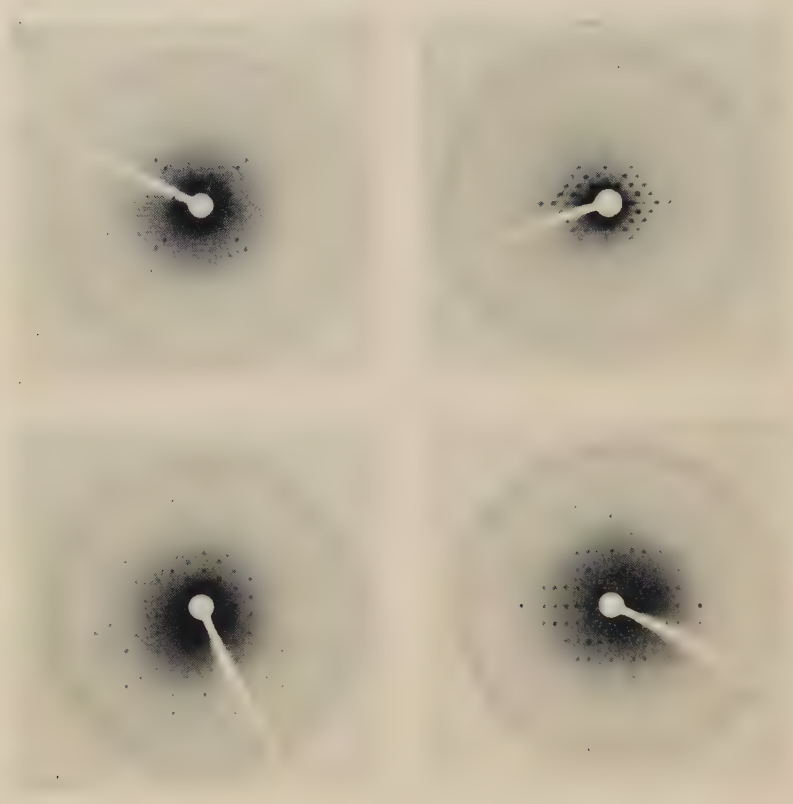

PLATE II. 6° precession X-ray diffraction photographs of ferritin B.

(a) X-ray beam parallel to [100]. [011] direction vertical.
(b) X-ray beam parallel to [110]. [1$\bar{1}$0] direction vertical.
(c) X-ray beam parallel to [111]. [1$\bar{1}$0] direction vertical.
(d) X-ray beam parallel to [112]. [1$\bar{1}$0] direction vertical.

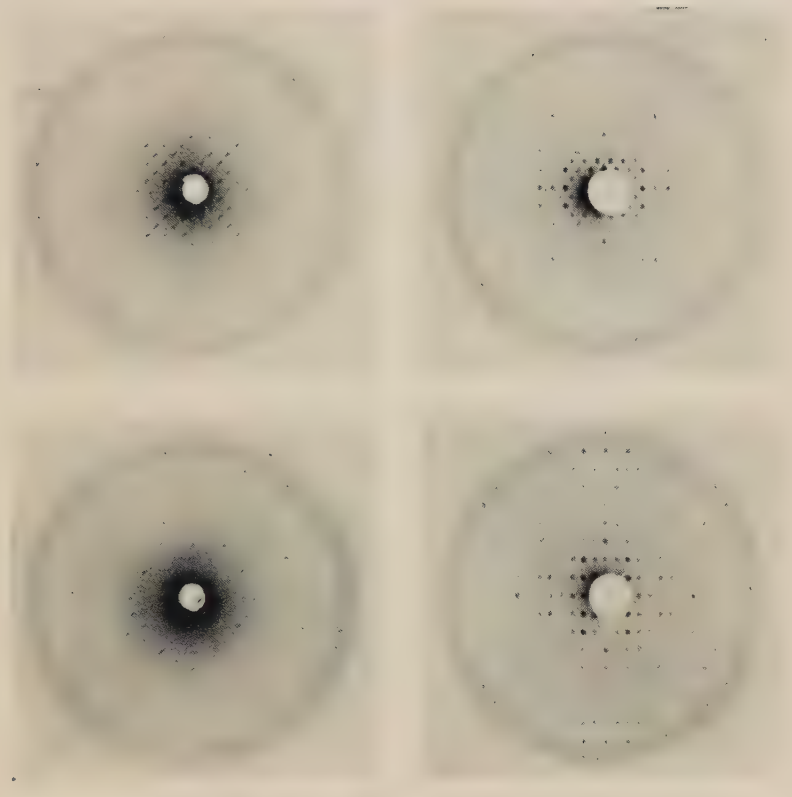

PLATE III. 6° precession X-ray diffraction photographs of apoferritin.

(a) X-ray beam parallel to [100]. [011] direction vertical.
(b) X-ray beam parallel to [110]. [1$\bar{1}$0] direction vertical.
(c) X-ray beam parallel to [111]. [1$\bar{1}$0] direction vertical.
(d) X-ray beam parallel to [112]. [1$\bar{1}$0] direction vertical.

PLATE IV. Optical diffraction pattern of sets of circular apertures placed at the vertices of sixteen snub-cubes. The snub cubes are arranged in four unit cells, space group $F432$, and projected down [100].

The optical diffraction pattern is on a scale about 1·27 times that of the X-ray photographs.

X-ray Data

Cell dimensions and symmetry

The work described in this paper is an extension of that reported earlier for ferritin (Hodgkin, 1949; Cowan, 1953) and now includes data for single crystals of apoferritin and for a second crystalline form of ferritin.

The crystals of horse spleen ferritin which were first studied (now designated ferritin A) were kindly supplied by Dr. Granick of the Rockefeller Institute for Medical Research. Many were quite large (0·5 mm or more) and were clearly optically anisotropic.

Two separate preparations of ferritin were made by the author from two horse spleens following Granick's (1942) method. In the first preparation only very small, or highly twinned, or poorly-formed crystals were obtained in several recrystallisations. No optical anisotropy was visible in any of the crystals and none has yet been examined by X-ray diffraction. From the second spleen larger, well-formed isotropic octahedra (0·2 mm across) were obtained by crystallisation from 5 % cadmium sulphate and these were used for the X-ray studies described below. A proportion of each of the two preparations was reduced to apoferritin by a modification due to Behrens & Taubert (1952) of the Granick & Michaelis (1943) method and the iron-free protein recrystallised from 5 % cadmium sulphate. Single crystals from the first spleen were again either small (0·1 mm or less) but well-formed colourless octahedra, or larger agglomerates. From the second spleen larger octahedra (0·3 mm) were grown from 5 % cadmium sulphate. All the apoferritin crystals examined were isotropic. X-ray diffraction photographs of crystals from both preparations indicated that the crystal structures were identical.

CuKα radiation was used for all the X-ray photographs: this is no doubt the cause of the rather heavy background scattering in the photographs of ferritin A and B, but the use of a copper target was unfortunately unavoidable.

Ferritin A. 2° oscillation photographs of large wet crystals of ferritin were taken with the X-ray beam parallel to the two-fold, three-fold and four-fold axes of the octahedron in succession. As expected from the optical anisotropy, the symmetry of these crystals is not cubic, but the X-ray diffraction photographs could be indexed on an orthorhombic unit cell closely related to the cubic cell of Fankuchen (1943). The orthorhombic unit cell has the dimensions

$$a = 130 \text{ Å}, \qquad b = 130 \text{ Å}, \qquad c = 184 \text{ Å}.$$

The a and b axes are both approximately equal in length to $c/\sqrt{2}$ and lie along directions parallel to the [110] and [1$\bar{1}$0] axes of the cube, while c is identical with the cubic axis. Reflections of indices $h00$, $0k0$, $00l$ were only observed when $h = 2n$, $k = 2n$ and $l = 2n$ respectively, there being no other systematic absences (although there was a marked tendency for $0kl$ reflections to occur only when $k + l = 2n$, there appeared to be occasional reflections for which this did not hold). The space group $P2_12_12_1$ was, therefore, formally deduced for the orthorhombic unit cell.

If, however, only low order reflections were taken into account, and a few reflections of very weak intensity were ignored, hkl reflections had the restriction $h + k + l = n$ and the orthorhombic cell was therefore apparently body-centred; moreover, the low order intensities exhibited marked pseudo-cubic symmetry, the cube edges of length 184 Å approximating to four-fold axes. This can be seen in Plate I (a) which is an X-ray photograph taken with the beam down one of the pseudo-four-fold axes, the $[001]_c$ axis. Reflections near the centre of the picture have indices h_ck_c0 and they

only occur with $h_c + k_c = 2n$, indices referring to the cubic cell being denoted by the suffix c. The strong reflections have both h_c and k_c even. A similar photograph taken with the beam down $[010]_c$ is shown in Plate I (b). In this case all the h_c0l_c reflections occur only with h_c even, while low order reflections with l_c odd are rather weak. The approximation to cubic symmetry for low orders and the departure from that symmetry for higher orders are well illustrated in Plates I (c) and (d), X-ray photographs taken with the beam parallel to the $[1\bar{1}0]_c$ and $[10\bar{1}]_c$ axes respectively. The similarity between these photographs for low orders is obvious, and yet there are also striking differences: thus, in Plate I (c) alternate layer lines perpendicular to the plane of the film are absent as compared to those in Plate I (d). On the other hand in Plate I (d) the reflections are centred, whereas in Plate I (c) departure from centring is quite marked. Hence it is deduced that for the "cubic" unit cell $h_ck_cl_c$ reflections are restricted to those for which $h_c + k_c = 2n$ (they can therefore be indexed in terms of the smaller, orthorhombic unit cell), while for low orders the restrictions that hkl reflections are observed only when $k + l = 2n$, $l + h = 2n$ are also approximately obeyed.

The orthorhombic space group $P2_12_12_1$ requires that there are at least four molecules in the unit cell. However, the symmetry of the low order reflections strongly suggests that, in fact, the molecules are arranged in face-centred cubic close-packing, and that the smaller orthorhombic unit cell is body-centred and contains only two molecules. If the space group were $P2_12_12$, $P2_122_1$, or $P22_12_1$, and the molecules contained a two-fold axis and were situated on unit cell dyads, two of the observed halvings would be explained, but not the third. Possible molecular arrangements are discussed in a later section of this paper. The spacing limit for wet crystal reflections was 3·4 Å.

Photographs of air-dried crystals of ferritin A were also taken. In this case not more than six orders of reflection could be seen (spacing limit 21 Å). Reflections could be indexed on an orthorhombic unit cell with cell constants $a = 111$ Å, $b = 111$ Å, $c = 156$ Å or a cube with $a = 156$ Å. The intensities exhibit cubic or near-cubic symmetry. Referred to the "cubic" cell $h_ck_cl_c$ reflections were observed only for planes with $h_c + k_c = 2n$, $k_c + l_c = 2n$, $l_c + h_c = 2n$, which implies face-centring. On the assumption that molecules are in contact in the air-dried crystals, the molecular diameter is 111 Å.

Ferritin B. Single wet crystal X-ray photographs of ferritin B were taken with a rotating anode tube as the source of X-rays and using a precession camera set for a 6° angle of precession. Crystals were examined with the X-ray beam parallel to the [100], [110], [111] and [112] directions of the octahedron. These photographs are reproduced in Plate II. The photographs of Plate II (b)–(d) were taken with three separate crystals; that of Plate II (a) was taken with the same crystal as for Plate II (d). The rapid fall off in intensity in Plate II (a) suggests that the crystal has deteriorated under the bombardment of X-rays. This photograph has not yet been repeated with a fresh crystal.

The X-ray photographs show that ferritin B is truly cubic over the range of reflections examined, with unit cell edge $a = 184$ Å and Laue symmetry $m3m$. The only systematic absences found were such that reflections of index hkl occurred only when the conditions $h + k = 2n$, $k + l = 2n$, $l + h = 2n$ were satisfied. This implies that the unit cell is face-centred. On the assumption that the cell contains a small number of molecules, it can be shown that this number must in fact be four. The space group is not uniquely determined from the reflection conditions, there being three or perhaps five possibilities. These are discussed in a later section in

relation to probable molecular structures. No photographs of air-dried ferritin B have yet been obtained.

Apoferritin. 6° precession photographs of wet apoferritin crystals were taken with a rotating anode X-ray tube, the beam being parallel to the [100], [110], [111] and [112] axes as shown in Plate III. Plate III (a) and (c) were obtained with the rather small crystals of the first preparation, Plate III (b) and (d) with larger ones from the second preparation. As in the case of ferritin B the unit cell is cubic with $a = 184$ Å. Again the Laue symmetry is $m3m$; $h + k$, $k + l$, $l + h$ are all even. For a unit cell with four molecules the systematic absences are again consistent with a choice of three or possibly five space groups of which $F432$ is most probable.

A number of X-ray photographs of air-dried apoferritin were taken with the crystal in various orientations. The symmetry and reflection conditions were the same as for the wet crystals although only six orders of reflection were observed. The unit cube edge a is equal to 158 Å.

An intermediate shrinkage state was also found for apoferritin, the reflections being indexed on a face-centred cube having $a = 173$ Å.

Molecular weights

The densities of wet and air-dried crystals of ferritin A measured by flotation in organic solvents (equilibrium method) were 1·357 and 1·497 g/cm³ respectively. Those of the smaller ferritin B wet crystals fell also mainly in the range 1·35–1·36 g/cm³ (equilibrium and gradient column methods) while the air-dried crystals appeared to have a rather higher density than those of ferritin A. The value of 1·45 g/cm³ reported by Fankuchen for *wet* crystals is very nearly as high as that obtained now for air-dried ferritin A, and is presumably due to the fact that only very small crystals were available for the earlier determination. An approximate initial density for the apoferritin crystals measured in a density gradient column was 1·16 g/cm³. The accuracy of this figure cannot be relied upon because the density increased rapidly over a short period, owing presumably to the rapid exchange of liquid of crystallisation with the organic solvents of the column. It is hoped that more accurate estimates can be made of the densities of ferritin B and apoferritin when larger crystals become available.

Loss of weight on drying the air-dried crystals of ferritin A was determined by Drs. Weiler and and Strauss to be 9·2 % by weight (*in vacuo* at 100°C). The corresponding data have not yet been obtained for ferritin B or apoferritin.

Using data for the air-dried crystals, the anhydrous molecular weight of ferritin A is calculated to be 776,000, if there are two molecules in the orthorhombic unit cell. This figure will be high, since cadmium sulphate of crystallisation has not been removed. From the difference between this figure and the weight per molecule in the wet unit cell, the volume of the wet crystals occupied by water can be calculated to be 53 % (assuming the density of water = 1). If the volume of cadmium sulphate solution of crystallisation is the same as that of the water (which will be very nearly true), and if the partial specific volume of the ferritin molecule in the crystal is the same as the apparent specific volume in dilute solution, 0·589 (Rothen, 1944), then the molecular weight of ferritin is 747,000 and the apparent density of the cadmium sulphate of crystallisation is 1·055.

The necessary data have not yet been obtained for the molecular weight determination of ferritin B, but since the wet crystal density is close to that of ferritin A, it is reasonable to assume that the molecular weights do not differ greatly.

Again in the case of apoferritin it has been shown that the cell dimensions of air-dried crystals are the same as those of ferritin A and it may be concluded that the molecular diameters are identical. Hence the volumes of interstitial liquid of crystallisation in wet crystals are also likely to be approximately the same. Using the values determined as above for volume and apparent density of the liquid of crystallisation in ferritin A, and the measured density of wet apoferritin crystals, a molecular weight of 563,000 is obtained for apoferritin. This value is, of course, subject to considerable error, but the very large discrepancy between it and the figure 465,000 obtained by sedimentation-diffusion (Rothen, 1944) can be taken to be due to the internal hydration of the molecule. The ultra-centrifuge molecular weight is an anhydrous one, based on the apparent specific volume of the anhydrous protein (0·747). If the partial specific volume of the apoferritin molecule in the wet crystals is also 0·747, then the X-ray data yield a figure of 462,000 for the anhydrous molecular weight, four molecules in the unit cell being assumed. From the discrepancy between this figure and that derived above it can be calculated that about 22 % of the molecular volume is occupied by water of hydration.

A rough estimate of the molecular weight of apoferritin can also be made directly from that of ferritin A. Thus, if the latter contains 23 % by weight of iron as FeOOH and if some phosphorus is also present in the micelle (Granick, 1946) then the protein component of ferritin A has a molecular weight 464,000. Again if the protein has a partial specific volume 0·747, then the micelle volume is 21 % of that of the whole molecule: this volume would correspond to that of a cube of side 54 Å or a sphere of radius 35 Å.

Discussion

The molecular structures of ferritin and apoferritin

From the X-ray data described above it has been concluded that the structures of apoferritin and of both forms of ferritin consist of an arrangement of four molecules in a face-centred cube of side 184 Å (wet crystals) or 156–158 Å (air-dried crystals). This arrangement is consistent with the electron microscope observations of Labaw & Wyckoff (1957) and gives an approximate molecular weight for apoferritin close to that obtained by Rothen (1944) from ultracentrifuge studies. A molecular diameter of 111–112 Å derived from the X-ray results for both ferritin A and apoferritin agrees closely with the values 100–109 Å obtained by electron microscopy (Farrant, 1954; Labaw & Wyckoff, 1957; Kuff & Dalton, 1957). Apoferritin and ferritin B appear to be isomorphous and to have full cubic symmetry. Ferritin A has the same lattice, but lower, orthorhombic, symmetry. The X-ray results will now be discussed in relation to the structures of the molecules. The simplest structure, that of apoferritin, will be taken first.

There are five cubic space groups consistent with the observed X-ray data for apoferritin: $F432$, $F\bar{4}3m$, $Fm3m$, $P4_232$ and $Pn3m$. Space groups containing mirror planes ($F\bar{4}3m$, $Fm3m$, $Pn3m$) can be rejected. Proteins are composed of amino acids all of which (apart from glycine) contain an asymmetric carbon atom in the L-configuration. Hence, the whole molecule, or any part of it which is built up from these units of like asymmetry, must be asymmetric. Protein crystals are, in turn, enantiomorphous. The space group $P4_232$ is strictly face-centred only for atoms in special positions and not for those in general positions. Molecules situated at these special positions would be expected to give rise to face-centring in low order spectra, the

condition breaking down at higher orders. In apoferritin the face-centring appears to be a general one and the space group can therefore be taken to be $F432$.

If the molecular weight of apoferritin molecules in the crystals is the same as that in solution (as determined by sedimentation-diffusion), the unit cell must contain four molecules. If the space group is $F432$ then the centres of these molecules must occupy special positions. Co-ordinates of these positions referred to the unit cell axes are either (0,0,0; $0,\frac{1}{2},\frac{1}{2}$; $\frac{1}{2},0,\frac{1}{2}$; $\frac{1}{2},\frac{1}{2},0$) or ($\frac{1}{2},\frac{1}{2},\frac{1}{2}$; $\frac{1}{2},0,0$; $0,\frac{1}{2},0$; $0,0,\frac{1}{2}$). It is a condition of the space group $F432$ that the four molecules at either set of special positions must have the cubic point group symmetry 432. This implies that the molecules are composed of a number of identical sub-units, related, in 432 symmetry, by four-fold, three-fold and two-fold axes, and that this number must be twenty-four or a multiple of twenty-four ($24n$).

The symmetry operations 432 on $24n$ identical sub-units will give rise to a roughly spherical shell of protein, and this is consistent with electron micrographs of ferritin which show that its shape is spherical or nearly spherical. Cubic point group symmetry has also been found in certain of the spherical plant viruses† (Caspar, 1956; Klug, Finch & Franklin, 1957). There are a number of different ways of arranging $24n$ equal sub-units to form a spherical shell. Of these the snub-cube, a semi-regular polyhedron, is perhaps the most attractive: it has 24 vertices, and sub-units placed at these positions form a closed shell having 432 symmetry. A drawing of a snub-cube is shown in Fig. 1. The optical diffraction pattern produced by an arrangement

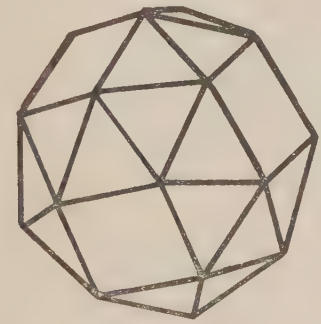

Fig. 1. Drawing of a snub-cube, a semi-regular polyhedron with 24 vertices.

of circular apertures at the vertices of sixteen snub-cubes projected down their four-fold axes (four unit cells) is reproduced in Plate IV. The most striking feature of this pattern is the concentration of intensity along the four-fold axes. A rather similar intensity distribution is also shown in the X-ray diffraction photograph of apoferritin shown in Plate III (a).

Although an arrangement of protein sub-units to form a structure with 432 symmetry (such as the snub-cube) does seem to fit the X-ray data for apoferritin

†The argument about sub-units and cubic symmetry was first used by Hodgkin (1949) in connection with the structure of plant viruses. She also pointed out that ferritin A was an example of a molecule adhering closely, but not strictly, to cubic symmetry. The same argument was later developed in more detail by Crick & Watson (1956). The plant viruses are in many ways analogous to ferritin; they contain a central core of ribonucleic acid, which may be removed in some cases and is surrounded by a shell of protein. Tomato bushy stunt and turnip yellow mosaic viruses have been shown to have the cubic point symmetry 532 (fully or in part) and hence to contain $60n$ sub-units.

reasonably well, it is possible that the observed four-fold symmetry of the lattice is a statistical effect produced by placing molecules with lower cubic point symmetry (23 or 532) at the lattice points with their two-fold axes parallel to the cubic axes, but orientated randomly with respect to those of neighbouring molecules. Molecules with 532 (or pseudo-532) symmetry in such an array would be expected to produce a diffraction pattern having maxima in the directions of the non-crystallographic five-fold axes. The existence in the X-ray diagrams of apoferritin of a number of strong reflections near such positions may support a structure of this type. On the other hand, optical transforms of snub-dodecahedra are rather weak in the regions of the cube axes as compared with the X-ray diffraction pattern of apoferritin, and this structure at least can probably be ruled out. A random array might also be expected to give rise to diffuse streaks in the diffraction pattern. These do not seem to occur in apoferritin, but the effects might be too weak to have been observed.

The molecules of apoferritin, then, are pictured as being constructed from a number of equal sub-units related by cubic point group symmetry; this symmetry is probably 432 ($24n$ sub-units), but may conceivably be 23 or 532 ($12n$ or $60n$ sub-units respectively). The addition to such a structure of the iron hydroxide micelles should now be considered, and an explanation sought for the differences between ferritins A and B. Once again, the simpler structure, that of ferritin B, will be discussed first.

The ferritin B lattice is isomorphous with that of apoferritin. It is therefore probable that there is a straight isomorphous replacement, that is to say that the protein shell is identical in structure and orientation (or that changes, if any, are minor ones which do not affect the symmetry) while there is a simple substitution of the water of hydration inside the apoferritin molecule by the micelle. This supposition is born out by the similarity in the diffraction patterns of apoferritin and ferritin B at intermediate angles, where it can be presumed that the micelle contributions are relatively small. This can be seen by comparing corresponding photographs in Plates II and III. The conclusion was reached above that the apoferritin molecules occupy special lattice positions and have the symmetry of these positions. Since the micelles lie at the centres of the molecules they must also belong to a cubic point group. However, the restriction of asymmetry does not necessarily apply to the inorganic ferric hydroxide structure, so that the point group may be either one of 432, $\bar{4}3m$ or $m3m$ (or perhaps 23, 532 or $m3$, with a random array of the type discussed for apoferritin above). It is conceivable that the protein has full cubic point symmetry, while the symmetry of the micelles is lower, the micelles being randomly orientated with respect to the protein. This would imply, however, a rather non-specific type of linkage between micelle and protein. Cubic symmetry would also be maintained if the micelles were structureless, although this is not consistent with electron microscope observations. The implications to be drawn from the presence of full four-fold cubic point symmetry in the micelles will be discussed below. Essentially similar conclusions can be arrived at if the micelles belong to one of the lower cubic point groups.

The only direct information available about the structure of the micelles is that obtained from electron micrographs, in which they appear frequently to be composed of four particles. Such an appearance might well result from four particles situated at the apices of a tetrahedron or eight particles at the corners of a cube, lying on a cube face. The [100] ferritin B diffraction pattern shown in Plate II (a) is very suggestive of a structure with a square projection such as would be obtained from either cube or tetrahedron. A third arrangement with cubic symmetry, that of six particles at the vertices of an octahedron, seems less likely and will not be considered further in

the present discussion. If the micellar form is that of either a cube or tetrahedron, and symmetries 432, $\bar{4}3m$ and $m3m$ are all possible, then certain deductions can be made about the number and symmetry of the sub-units: these are summarised in Table 1. Thus, for example, if the point group symmetry of the micelle is 432 and there are eight sub-units, then the crystal class to which the iron hydroxide structure belongs must be the trigonal one, 3, whereas if there are four sub-units at the apices of a tetrahedron, point symmetry $\bar{4}3m$, then the iron hydroxide sub-units must have trigonal symmetry $3m$. The actual structure of the iron hydroxide in the micelle is not known for certain. However, it has been reported that ferritin can be synthesised from apoferritin, ferrous ions and air in bicarbonate buffer at pH 7·4 (Bielig & Bayer, 1955*b*; Loewus & Fineberg, 1957). Oxidation of ferrous ions in aqueous solution normally leads to either the α-(goethite) or γ- (lepidocrocite) FeOOH structures. Under the above experimental conditions α-FeOOH would be expected to result. Both the goethite and lepidocrocite structures belong to the orthorhombic crystal class mmm. If the micellar iron hydroxide has either of these structures,† and the four-particle tetrahedron or eight-particle cube arrangement, then in neither case can the single particle be the fundamental sub-unit, which must be smaller. The simplest arrangement possible would be that with $\bar{4}3m$ symmetry and twelve sub-units. The latter could be grouped in threes round the three-fold axes so as to

Table 1

Micelle point group symmetry	Form	Number of identical sub-units	Point group symmetry of sub-units
432	cube	8	3
		24	1
$\bar{4}3m$	tetrahedron	4	$3m$
		12	m
		24	1 (two mirror-image forms)
$m3m$	cube	8	$3m$
		24	m
		48	1 (two mirror-image forms)

give four separate particles. Although such a structure may seem unduly complicated, it is of interest to note that there is also evidence from electron microscopy that the fundamental sub-unit may be smaller than any of the 27 Å diameter particles of the micelle (Richter, 1957).

Suppose, then, that the structure discussed for ferritin B above, a molecule with 24 (or $24n$) protein sub-units in 432 symmetry and 12 ferric hydroxide sub-units in $\bar{4}3m$ symmetry or twenty-four FeOOHsub-units in 432 or $m3m$ symmetry, is the correct one, what then is the relation between ferritin B and ferritin A? It is conceivable that the protein sub-units are arranged differently or that the amount of iron differs in the two molecules. However, it is not necessary to seek an explanation in terms of radical

†Richter (1958*b*) states that the inference that the micelles consist of iron hydroxide has been confirmed by electron diffraction but does not give details.

differences in molecular composition. There are two other main possibilities. One is that the molecules of ferritin A and B are identical, but have different orientations in the two crystal structures; the other that the protein shells are arranged in precisely the same manner while the micelles are disposed differently with respect to the protein.

The X-ray diffraction pattern of ferritin A shows that, although markedly pseudo-cubic, the symmetry is actually orthorhombic. Referred to the orthorhombic unit cell, the X-ray data exhibit halvings along all three axes: two of these could be accounted for if the space group were $P2_12_12$, $P2_122_1$ or $P22_12_1$, the unit cell containing two molecules lying on the two-fold axes (and therefore themselves containing dyads). The third axial halving (a non-space group halving) and the pseudo-cubic symmetry of the diffraction pattern would be produced if the molecules had cubic point symmetry and were centred at $\frac{1}{4}$ and $\frac{3}{4}$ along the unit cell two-fold axes, but were rotated so that their other symmetry axes were not coincident with lattice directions. Thus molecules with 432 symmetry have two-fold axes parallel to cube face-diagonals and therefore to the orthorhombic a and b axes; if they were rotated so that only one of their dyads lay along a or b the required symmetry would be produced. The situation is similar if the molecules have point group 23 or 532 and c is the unit cell dyad.

The cubic symmetry of the crystals would be destroyed, on the other hand, if the symmetry of the individual molecules were lowered by a re-orientation of only *part* of the molecule, e.g. if the micelle had point group $\bar{4}3m$ and was turned so that *one* of its two-fold axes was parallel to either a or b. In this situation those reflections forbidden by cubic symmetry, but which were observed in ferritin A, would be due entirely to the micelles.

It is at present difficult to decide between the various alternatives before a detailed study has been made of the intensities of the X-ray reflections. At low angles the X-ray diffraction patterns of ferritin A and B are broadly similar but have quite important differences in detail, suggesting that the micellar arrangement may be substantially different. At intermediate angles the diffraction pattern of ferritin A, although not identical with those of either ferritin B or apoferritin, has maxima at roughly the same positions, suggesting that the protein orientation may be the same or not markedly different. The identity of lattice repeats might also be cited in favour of a structure in which the protein shells, which govern intermolecular contacts, are arranged in the same way in all three crystals. This argument would not have much weight, however, if the protein shell were nearly spherical. If the protein structures are isomorphous, on the other hand, then there must be two species of ferritin molecule which crystallise selectively. Such molecules would, however, differ only in internal structure and would not be distinguishable by properties depending on their external shape or surface structure—immunological reactions, viscosity and electrophoretic mobility. A detailed study of the three crystals is now being made and it is hoped to be able to decide between the structures outlined in the preceding paragraphs and perhaps others in the near future.

Molecular structure and function of ferritin

An ultimate aim of the study of the structure of a protein is to relate this structure to the physiological functions of the molecule. Ferritin has three such functions (Mazur, 1956):

(1) as an iron storage protein—the iron is held in reserve until required, when it is released, e.g. for the synthesis of haem.

(2) as a "vasodepressor"—small quantities of ferritin will inhibit the response of the smooth muscle cells of the precapillary blood vessels to the topical application of adrenalin.

(3) as an anti-diuretic agent—by stimulating the secretion of the anti-diuretic hormone and hence causing the resorption of water.

All three activities appear to require the presence of ferrous iron. Thus ferritin has been synthesised *in vitro* from apoferritin and ferrous ions by atmospheric oxidation (Bielig & Bayer, 1955*b*; Loewus & Fineberg, 1957) whereas ferric ions have little effect (Loewus & Fineberg, 1957). It would thus appear that the iron is oxidised only as it enters the molecule where it is incorporated in the form of the iron hydroxide micelle. It is released as ferrous iron. The highly-ordered structure found for ferritin A suggests that the micelle is held inside the protective protein shell at quite specific sites (although the existence of ferritins A and B *may* mean that there are two alternative sites). One of the objections to a structure with the micelle *inside* the molecule has been the difficulty of imagining how the iron could pass through the protein shell. In the type of structure suggested above—a semiregular polyhedron formed by the assembly of equal sub-units—the surface is not completely covered. On certain faces there may be quite large gaps; these may provide a convenient channel for the passage of iron in and out of the molecule.

The other activities of ferritin also appear to depend on the state of oxidation of the molecule: they require the presence of a small amount of ferrous iron. Thus the ferric iron of the micelle is thought to be in equilibrium with a small quantity of iron at the surface of the molecule; when the latter is in the ferrous state it is chelated to sulphydryl groups and thus stabilised against autoxidation. When the sulphydryl groups are oxidised or blocked, the ferrous iron is oxidised and the ferritin loses its activity. If apoferritin sulphydryl groups are reacted with *p*-chloromercuribenzoate and the product crystallised, it may be possible by X-ray diffraction to determine whether the highly scattering mercury atoms, and hence the sulphydryl groups, are located at the surface of the molecule.

It is thus hoped that a further and more detailed X-ray study may lead to the determination of the molecular architecture of the ferritin molecule and provide some explanation of its functions *in vivo*. The existence of the three crystalline forms, ferritin A and B and apoferritin, should facilitate this task.

The work on ferritin A was carried out under the supervision of Dr. Dorothy Hodgkin at the Chemical Crystallography Laboratory, Oxford, being part of a research programme for the degree of Doctor of Philosophy. This work was supported by grants from the Rockefeller and Nuffield Foundations. I am deeply grateful to Dr. Hodgkin for many ideas and suggestions and to Dr. Granick for supplying the crystals of ferritin A. The work on ferritin B and on apoferritin, which is now continuing, is aided by a research grant from the Medical Research Council, London. Precession photographs of apoferritin and of ferritin B were taken at the Royal Institution, London, and I am very much indebted to Sir Lawrence Bragg for kindly making facilities available, and also to Drs. A. C. T. North and D. W. Green for unstinting assistance during brief visits to the Davy-Faraday Laboratory. I should also like to thank Dr. D. Caspar for helpful discussion and Dr. A. Klug for making a number of suggestions and for supplying me with optical transforms of the snub-dodecahedron; Dr. C. A. Taylor for kindly photographing the transform of a snub-cube, and Miss J. Gurr for making photographic reproductions of the X-ray diagrams. Finally, but by no means least, I must acknowledge the courtesy of Professor J. H. Gibson for kindly providing me with laboratory facilities at the Department of Biochemistry, the University of Sheffield.

REFERENCES

Behrens, M. & Taubert, M. (1952). *Hoppe-Seyl. Z.* **290**, 156.
Bessis, M. & Breton-Gorius, J. (1957). *C. R. Acad. Sci., Paris*, **245**, 1271.
Bielig, H. J. & Bayer, E. (1955*a*). *Naturwissenschaften*, **42**, 466.
Bielig, H. J. & Bayer, E. (1955*b*). *Naturwissenschaften*, **42**, 125.
Caspar, D. L. D. (1956). *Nature*, **177**, 476.
Cowan, P. M. (1953). *D. Phil. Thesis, Oxon.*
Crick, F. H. C. & Watson, J. D. (1956). *Nature*, **177**, 473.
Fankuchen, I. (1943). *J. Biol. Chem.* **150**, 57.
Farrant, J. L. (1954). *Biochim. biophys. Acta*, **13**, 569.
Granick, S. (1942). *J. Biol. Chem.* **146**, 451.
Granick, S. (1946). *Chem. Rev.* **38**, 379.
Granick, S. & Michaelis, L. (1943). *J. Biol. Chem.* **147**, 91.
Hodgkin, D. C. (1949). *Cold Spr. Harb. Symp. Quant. Biol.* **14**, 65.
Klug, A., Finch, J. T. & Franklin, R. E. (1957). *Biochim. biophys. Acta*, **25**, 242.
Kuff, E. J. & Dalton, A. L. (1957). *J. Ultrastructure Res.* **1**, 62.
Labaw, L. W. & Wyckoff, R. W. G. (1957). *Biochim. biophys. Acta*, **25**, 263.
Laufberger, M. (1937). *Bull. Soc. Chim. Biol., Paris*, **19**, 1575.
Loewus, M. W. & Fineberg, R. A. (1957). *Biochim. biophys. Acta*, **26**, 441.
Mazur, A. (1956). *Essays in Biochemistry* (S. Graff, Ed.). p. 198. New York: John Wiley and Sons, Inc.
Mazur, A., Baez, S. & Shorr, E. (1955). *J. Biol. Chem.* **213**, 147.
Mazur, A., & Shorr, E. (1950). *J. Biol. Chem.* **182**, 607.
Michaelis, L. (1947). *Advanc. Protein Chem.* **3**, 53.
Novikoff, A. B., Beaufay, H. & De Duve, C. (1956). *J. Biophys. Biochem. Cytol.* **2** Suppl. 2, 179.
Palade, G. E. & Siekevitz, P. (1956). *J. Biophys. Biochem. Cytol.* **2**, 171.
Richter, G. W. (1957). *J. Exp. Med.* **106**, 203.
Richter, G. W. (1958*a*). *J. Biophys. Biochem. Cytol.* **4**, 55.
Richter, G. W. (1958*b*). *Bull. N. Y. Acad. Med.* **34**, 134.
Rothen, A. (1944). *J. Biol. Chem.* **152**, 679.

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LETTERS TO THE EDITOR

Gene Replication and Mutation Induction in Bacteria†

Watson & Crick (1953) have suggested a direct method of deoxyribonucleic acid (DNA) replication through specific hydrogen-bond attraction of complementary purine and pyrimidine bases to the separated strands of the double-stranded DNA macromolecule. Recently Stent (1958) proposed a scheme for DNA replication which has as its main feature the passing of genetic information from the parent DNA macromolecule through ribonucleic acid (RNA) and possibly protein to the daughter DNA macromolecule. While an appreciation of the Stent model as opposed to the Watson-Crick model for DNA replication will not be attempted here, we should like to present some recent observations of the UV-induced mutation process and macromolecular synthesis in *Escherichia coli* which possibly should be considered in the evaluation of various models of DNA replication.

We have advanced the hypothesis that nucleic acid precursors altered *in vivo* by ultraviolet radiation constitute chemical intermediates in UV-induced mutation (Haas & Doudney, 1957). We have further demonstrated (Doudney & Haas, 1958) that *Escherichia coli* exposed to UV and held under certain conditions limiting to protein synthesis shows an immediate enzymatic decline in mutation frequency. In cultures held under conditions favoring protein synthesis the mutation frequency does not decline; rather the potential mutations are "stabilized" in the cell. Subsequent to "stabilization" the mutations are fixed in the cell by a process which we call "mutation fixation" (MF).

We have previously suggested that MF may involve ribonucleic acid synthesis (Doudney & Haas, 1958). To test this hypothesis we carried out the experiment shown in Fig. 1 in which MF was compared to the macromolecular synthetic activities of a culture of *E. coli* strain WP2 (tryptophan-requiring). MF was determined by treatment of the irradiated culture of *E. coli* strain WP2 with chloramphenicol for 45 min following the indicated post-irradiation incubation interval (see Doudney & Haas, 1958, for details of the experimental procedure). Extraction procedures for nucleic acid and protein were those of Ogur & Rosen (1950). RNA was determined by the method of Vishner & Chargaff (1948); DNA by the method of Burton (1956); and protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

It is evident that MF is closely correlated with RNA synthesis in the culture. MF is initiated at the time RNA synthesis starts and has attained its maximum level at the time the amount of RNA has doubled. This particular experiment was chosen for presentation here since for some unknown reason the rate of RNA synthesis shows a decrease during the 35–45 min period of post-irradiation incubation; a corresponding change in the course of MF is observed. It is interesting to note that this reduction in rate of RNA synthesis during the post-irradiation incubation period (35–45 min) is reflected in a reduction in rate of protein synthesis at 45–60 min. This suggests a direct sequential relation of RNA synthesis and protein synthesis.

It should be pointed out, however, that the change in rate of RNA synthesis demonstrated is not our usual finding here; rather RNA synthesis proceeds in a linear

†This work was supported in part by National Institutes of Health Grant C(3323).

fashion once initiated. We have retarded RNA synthesis for a period by addition of 5-hydroxyuridine to the medium and as expected we find that MF is retarded accordingly. Both RNA synthesis and MF resume on reversal of the 5-hydroxyuridine inhibition with uridine.

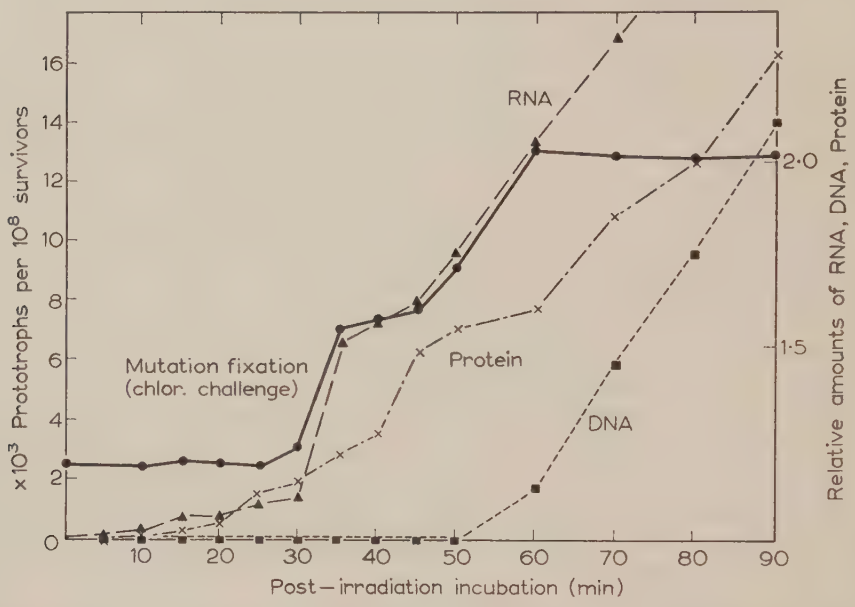

FIG. 1. The relation of "mutation fixation" as demonstrated by "chloramphenicol challenge" to the synthesis of RNA, DNA and protein in an UV-exposed culture of *Escherichia coli* strain WP2. UV exposure was for 10 sec at 30 cm (964 ergs/mm² below 2,800 Å). The unexposed control suspension contained $6{\cdot}5 \times 10^8$ colony-forming cells per ml. The exposed culture contained $5{\cdot}1 \times 10^8$ colony-forming cells per ml. The bacteria were incubated after radiation exposure at a 1:5 dilution in minimal medium supplemented with 2 mg/ml. casein hydrolysate and 0·2 mg/ml. DL-tryptophan. After the indicated interval of incubation chloramphenicol was added to a final concentration of 20 μg/ml. and the culture incubated for an additional 45 min ("chloramphenicol challenge"). For details of the procedure followed for culture growth, radiation exposure, post-irradiation treatment and mutation assay see Doudney & Haas (1958).

It is evident from this experiment that protein synthesis is not *directly* involved in MF, being separated from it in time. Further it appears that DNA synthesis is not directly involved in MF; no net DNA synthesis is observed here until the MF process is complete. Since DNA synthesis commences at about the time that the maximum level of MF is attained it may be that the initiation of DNA synthesis limits further MF though we have no evidence to suggest that this is necessarily the case.

Further experiments demonstrate that mutation fixation, as measured by loss with incubation of susceptibility of the "potential mutation" to photoreversal, is closely correlated to the MF demonstrated by chloramphenicol treatment. This lends significance to the view that MF is the manifestation of the macromolecular synthetic event directly involved in the irreversible establishment of those changes which constitute mutation. It would be expected that the last possible point of action of a physical agent such as light in preventing mutation would be just prior to the occurrence of such an irreversible macromolecular change.

The evidence suggests that following alteration of the nucleic acid precursors by UV, the primary mutagenic event involves the synthesis of RNA from these precursors. Thus the possibility exists that RNA synthesis is intermediate to synthesis of

DNA, and that RNA (and possibly protein) actually mediates transfer of "information" from parent to daughter genic DNA. This is not to suggest that all the cellular RNA synthesized during the course of MF is involved in DNA replication. Rather the minor RNA synthesis involved in DNA replication may be correlated with gross RNA synthesis in the cell under our experimental conditions.

It is evident that the sequence of macromolecular syntheses which our data suggests does not harmonize with the model of DNA replication proposed by Watson & Crick.

Stent (1958) has presented a scheme of DNA replication in which the double-stranded helical DNA molecule serves as a template, through complementary hydrogen-bonding of the purine and pyrimidine bases, for synthesis of a nucleoprotein molecule consisting of one ribonucleic acid strand and a protein backbone. He suggests that the building blocks, or monomers, for the synthesis of the third chain could be an amino acid-ribotide complex, so that the growth of a polypeptide backbone chain could follow the growth of the polyribotide chain. This chain then would unwind and separate. The genetic information of the parent DNA double helices would now reside in a single RNA chain. The mechanism for the synthesis of replica DNA would be the inverse of that by which the single RNA strand has been formed by the parental DNA molecule. Thus a complementary pair of deoxyribonucleotides is attracted by the single RNA base through the formation of specific hydrogen bonds. This is followed by polymerization into DNA.

The similarity of our experimentally formulated scheme of UV-induced gene mutation (and the macromolecular interrelations in gene replication which it suggests) to the theoretical scheme of Stent is obvious. The possibility exists that the process of stabilization of the potential mutation which our data suggests actually involves the specific association of amino acid-nucleotide monomers with the DNA double helix as proposed by Stent. We have previously suggested that the "stabilization" of potential mutations involves an amino acid-nucleotide complex (Doudney & Haas, 1958). Furthermore, the process of "mutation fixation" may be the polymerization of the nucleotide monomers into RNA. Further exposition of Stent's model in the light of our experimental findings will not be attempted here; however, it appears necessary that some such scheme for DNA replication involving intermediate RNA synthesis must hold if the experimental data we have obtained concerning the process of UV-induced mutation is to be explained at the level of macromolecular synthesis.

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Burton, K. (1956). *Biochem. J.* **62**, 316.
Doudney, C. O. & Haas, F. L. (1958). *Proc. Nat. Acad. Sci., Wash.* **44**, 390.
Haas, F. L. & Doudney, C. O. (1957). *Proc. Nat. Acad. Sci., Wash.* **43**, 871.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265.
Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.
Stent, G. S. (1958). *Advances in Virus Research*, **5**, 95.
Vishner, E. & Chargaff, E. (1948). *J. Biol. Chem.* **176**, 703.
Watson, J. D. & Crick, F. H. C. (1953). *Cold Spr. Harb. Symp. Quant. Biol.* **18**, 123.

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The Icosahedral Form of an Adenovirus

Valentine & Hopper (1957) suggested that adenovirus might have a polyhedral shape since electron micrographs showed phosphotungstic acid-stained virus particles to be hexagonal in outline. The same appearance was found in carbon replicas, but shadowed air-dried preparations showed roughly spherical particles with only a slight suggestion of angularity in the shadows. Valentine & Hopper suggested that the particles were either rhombic dodecahedra or icosahedra, but could not draw a precise conclusion. In the case of the much larger *Tipula* Iridiscent Virus ($\sim$ 1300 Å in diameter), Williams & Smith (1958) were able to deduce that the form was icosahedral from the shapes of the shadows of particles shadowed at different angles.

In this communication we wish to report an electron microscopical study of adenovirus, using the negative staining technique of Brenner & Horne (1959); this study shows that the particles are icosahedral, and also permits the resolution of subunits in the virus so that an exact model of the particle can be built. We also present some preliminary findings with herpes virus, which may have the same structure.

Adenovirus, type 5, was grown in HeLa cells for 5 days. The virus was harvested from the cells by 4 cycles of freezing and thawing, followed by low speed centrifugation to remove debris. The supernatant was centrifuged for 1 hr at 60,000 *g*, and the pellets were resuspended in water. Herpes virus, strain HFEM, was grown in HeLa cells following the procedure of Farnham & Newton (1959). The infected cells were disrupted with a microblendor, centrifuged at low speed, and the virus sedimented from the supernatant by centrifugation at 40,000 *g* for 1 hr. The pellets were suspended in distilled water. The viruses were prepared for electron microscopy by mixing equal volumes of the virus suspension and 2 % phosphotungstic acid previously adjusted to pH 7 with N-KOH. The mixture was then sprayed on to carbon-coated electron microscope grids (Brenner & Horne, 1959). The specimens were examined in a Siemens Elmiskop electron microscope, and photographed at instrumental magnifications of $\times$ 40,000 and $\times$ 80,000 using double condenser illumination. The illuminating probe was reduced to 15 microns in diameter when working at these magnifications. Through-focal series of photographs were taken to eliminate any possible structure produced by interference and phase contrast effects.

Although the virus suspensions are not pure, the characteristic particles of adenovirus, approximately 700 Å in diameter, can easily be detected, embedded in the electron-dense background of phosphotungstate. A low power view is shown in Plate I (*a*), from which it may be seen that the particles have a subunit structure. Plate I (b) is an electron micrograph of two particles at a higher magnification, and shows that the subunits are not arranged at random but have a definite pattern. The nature of the arrangement can be easily deduced from the virus particle shown in Plate II (a). In this orientation the particle has a roughly hexagonal outline and is clearly facetted, each facet being an equilateral triangle with a side consisting of six subunits. The centre-to-centre distance between subunits is 70 Å, and the length of a side is 420 Å. The presence of triangular facets immediately excludes a dodecahedral

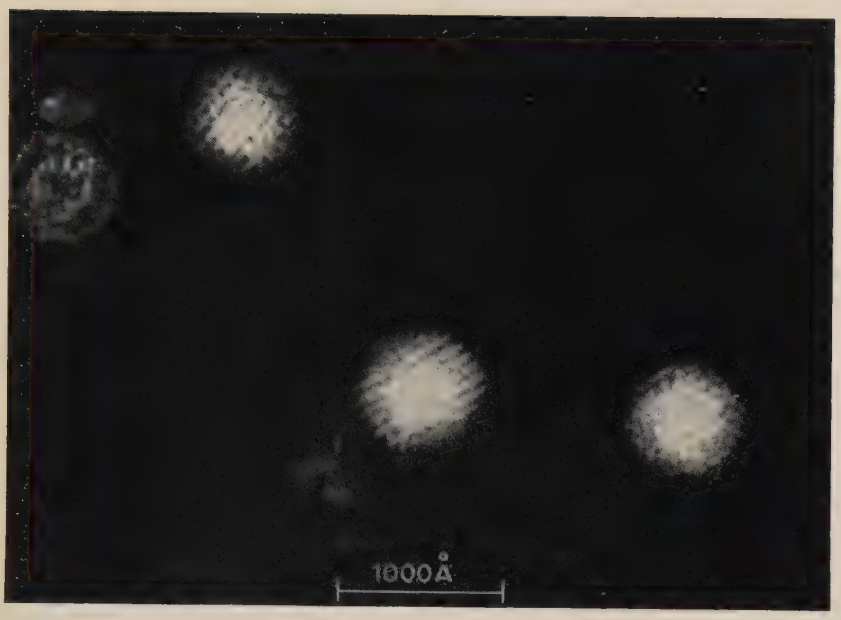

PLATE I(a). Adenovirus particles embedded in phosphotungstate, at low magnification (× 220,000).

PLATE I (b). Two adenovirus particles at high magnification (× 800,000).

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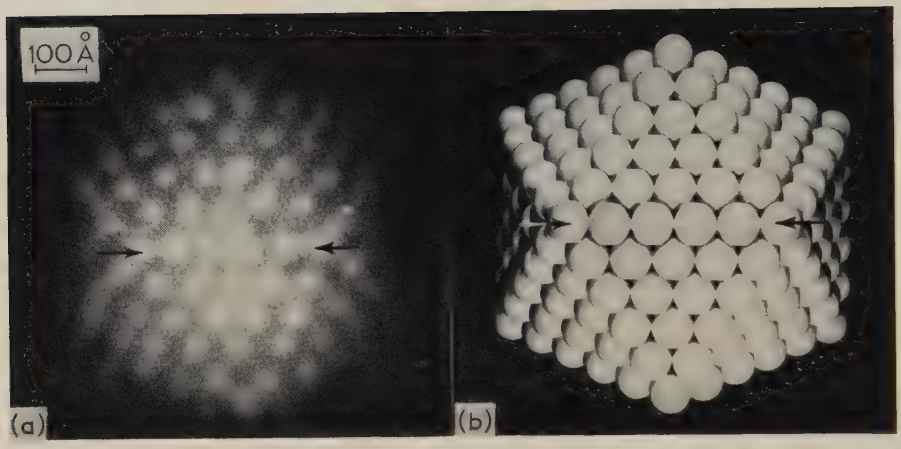

PLATE II. A. Adenovirus particle, at high magnification (× 700,000). B. Photograph of model of icosahedron in same orientation. The arrows mark subunits at the vertices which lie on five-fold rotational symmetry axes.

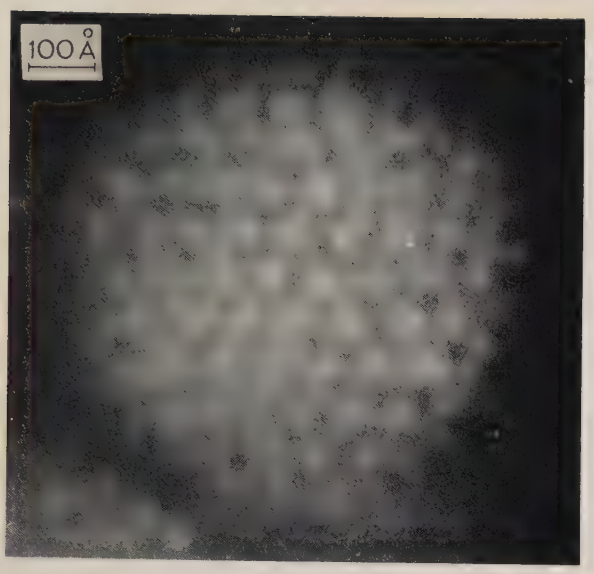

PLATE III (a). Adenovirus particle at high magnification (× 850,000).

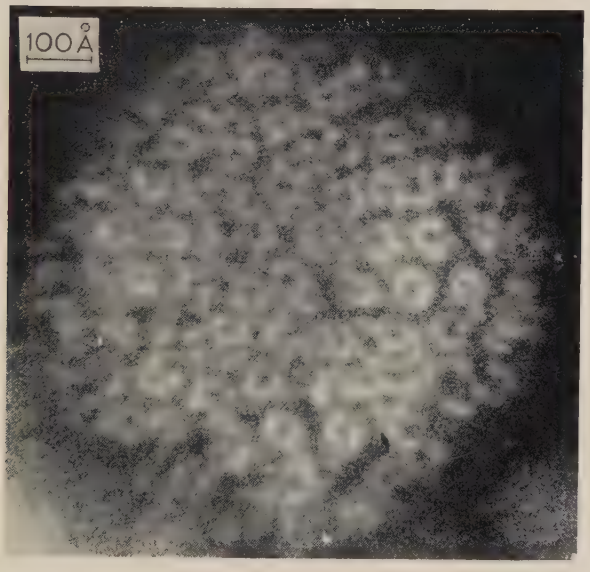

PLATE III (b). Herpes virus at the same magnification as the adenovirus particle in Plate III (a), showing hollow subunits. (× 850,000).

form and suggests that the particle is an icosahedron. A model of an icosahedron with a side of six subunits has been built from table tennis balls, and Plate II (b) is a photograph of this model standing on an edge. The arrangements of the subunits in the virus particle and in the model are identical. It should be particularly noted that, in both cases, the subunits which occupy the apices of the faces lie on five-fold symmetry axes and are surrounded by five other subunits, while those on edges or faces are surrounded by six other subunits. The virus particles shown in Plate I (b) may also be readily interpreted as icosahedra orientated in different ways.

Herpes virus shows a similar subunit structure but the subunits appear to be hollow and ring-shaped when compared with adenovirus (Plates III(a) and III (b)). The preparations are, as yet, not good enough to demonstrate unequivocally the exact shape of the virus as the structure seems to be very easily deformed. It is possible that this virus may also be icosahedral.

The idea that small spherical virus particles are built up of subunits related by cubic symmetry elements was originally suggested by Hodgkin (1949) and developed by Crick & Watson (1956). Of the three possible cubic symmetry point groups considered by Crick & Watson, 532 symmetry, corresponding to the symmetry elements of a dodecahedron or an icosahedron, was found in tomato bushy stunt virus by Caspar (1956). The presence of this type of symmetry demands that the number of subunits in the virus should be 60 or a multiple thereof. Klug, Finch & Franklin (1957) have recently presented evidence for 532 symmetry in turnip yellow mosaic virus and have suggested that the virus shell is built of 60 asymmetric units, 60 Å in diameter, placed at the vertices of a snub dodecahedron. It is important to emphasize that the predictions of Crick & Watson concern the *symmetry elements* in a virus particle and not its *shape*. The presence of 532 symmetry implies only that a defined packing arrangement is used over and over again, so that each identical subunit has exactly the same neighbours as any other; it does not imply that the shape of the virus must necessarily be dodecahedral or icosahedral. This distinction is brought out in the case of adenovirus. The shape of the virus is clearly icosahedral and the number of subunits is 252 which is not a multiple of 60 as required for 532 symmetry. Furthermore, it can easily be seen that the subunits do not all have the same packing arrangement and, in fact, three different kinds of units with different neighbours may be distinguished: (*a*) the twelve units at the vertices which have only five neighbours, (*b*) the four units occupying each of the thirty edges of the icosahedron which have six neighbours in different planes and (*c*) the remaining 120 units which occupy the faces and which have six neighbours all in the same plane. Thus the icosahedral shape could be obtained if three chemically different subunits were used to build the outer shell. On the other hand, it is possible that the subunits are all identical and that the shape is determined by the symmetry of an internal structure which may be the nucleic acid of the virus itself or a nucleoprotein particle.

The general comment may be made that large polyhedral viruses built of many subunits are most likely to have triangular facets since this permits hexagonal packing of the subunits in a facet. This close packing cannot be achieved with a pentagonal facet and, for this reason, dodecahedral viruses are unlikely to be found.

We may conclude that adenovirus is an icosahedron, with a shell constructed from 252 subunits, each triangular facet having a side of six subunits. The maximum diameter of the subunits as estimated from the centre-to-centre distance is 70 Å, and each may therefore represent a single protein molecule. Herpes virus also shows a subunit structure but the subunits are ring-shaped.

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REFERENCES

Brenner, S. & Horne, R. W. (1959). *Biochim. biophys. Acta*, in the press.
Caspar, D. L. D. (1956). *Nature*, **77**, 476.
Crick, F. H. C. & Watson, J. D. (1956). *Nature*, **177**, 473.
Farnham, A. E. & Newton, A. A. (1959). *Virology*, in the press.
Hodgkin, D. C. (1949). *Cold Spr. Harb. Symp. Quant. Biol.* **14**, 65.
Klug, A., Finch, J. T. & Franklin, R. E. (1957). *Biochim. biophys. Acta*, **25**, 242.
Valentine, R. C. & Hopper, P. K. (1957). *Nature*, **180**, 928.
Williams, R. C. & Smith, K. M. (1958). *Biochim. biophys. Acta*, **28**, 464.

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